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An Investigation of Enzymes Capable of Activating Cannabinoid Prodrugs

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AN INVESTIGATION OF ENZYMES CAPABLE OF ACTIVATING CANNABINOID PRODRUGS

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has been approved as meeting the requirements for the Degree of Master of Science in the College of Natural and Health Sciences in the Department of Chemistry and Biochemistry

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ABSTRACT

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The plant *Cannabis sativa* contains a class of terpenophenols called cannabinoids which, in their active form, have been shown to have antiproliferative, anti-metastatic, anti-angiogenic, and pro-apoptotic effects on several types of cancer cells both *in vitro* and *in vivo.* Cannabinoids have been shown to not exert this apoptotic activity in healthy cells and they do not elicit the usual side effects seen with conventional chemotherapies. However, naturally occurring prodrugs from *Cannabis* must first be converted into the active form to induce the apoptotic and other physiological activities. Although humans do not possess the enzyme(s) necessary for this conversion, several different microbial species have been shown to have enzymes capable of catalyzing reactions similar to that required for activation of the cannabinoid prodrug(s).

This research focused on the isolation of the prodrug cannabidiolic acid (CBDA) from *Cannabis* and screening of various microbial cell lines capable of enzymatic conversion of CBDA to the active drug cannabidiol (CBD). This involved the development of a preparative HPLC method for isolation of CBDA and an analytical HPLC method for the monitoring of enzymatic prodrug conversion. Seven microbial species were screened for CBDA decarboxylation activity of the prodrug from *Cannabis*.

The cells were incubated with CBDA, and the substrate and resulting product(s) from incubation were simultaneously extracted from the incubated cell suspension at different time intervals and analyzed using HPLC. The Gram-negative bacterium *Pseudomonas putida* was identified to possess this enzymatic activity and will be an ideal species for gene isolation and further screening of the target enzyme. The results from this research provide an alternative to the traditional mode of activating cannabinoid pro-drugs and a new way to potentially control and monitor the effective dose of cannabinoids being administered.

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CHAPTER I

INTRODUCTION

With origins that date back to ancient times, many botanical extracts utilized as folk remedies have now been developed into modern day medicine. Acetylsalicylic acid (e.g., aspirin) was synthesized from a compound that came from the bark of Willow trees (*Salix alba*) which was used by many ancient cultures as a pain remedy before the discovery of salicylic acid as the naturally occurring active drug (Mahdi, Mahdi, Mahdi, & Bowen, 2006). More recently the plant *Cannabis sativa,* which has long been cultivated and harvested for its medicinal qualities, has been of interest to the scientific community. The first accounts of *Cannabis* used medicinally come from the world's oldest pharmacopoeia, the *Pên-tsao Ching*, which was compiled in 4,000 B.C. based upon oral traditions (Li, 1974). Despite its historic prevalence, however, the primary active compounds in the plant *Cannabis sativa,* phytocannabinoids, were not initially characterized until 1964 (Gaoni & Mechoulam, 1964).

At least 150 different phytocannabinoid compounds, considered terpenophenols, have been identified from *Cannabis sativa* over the past few decades (ElSohly & Gul, 2016). Of these compounds, the most abundant are Δ^9-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), cannabigerolic acid (CBGA) (Figure 1), Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN), and cannabigerol (CBG) (Figure 4) (Backer et al., 2009).

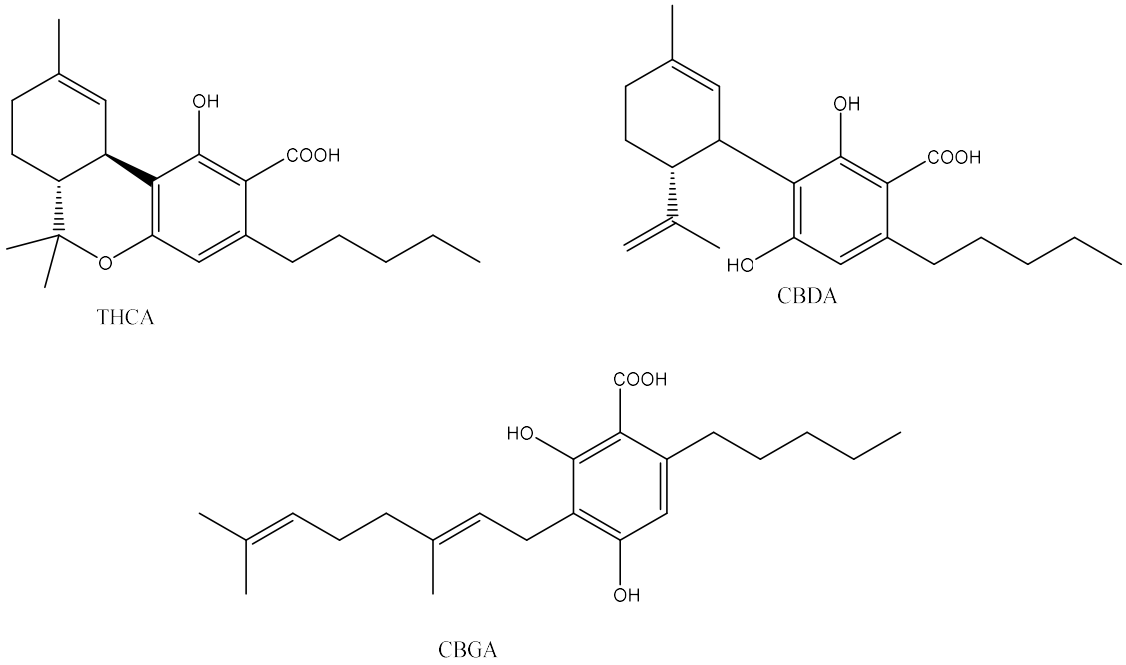


Figure 1: Structures of Δ^9-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabigerolic acid (CBGA).

Initially, due to their lipophilic nature, it was assumed that cannabinoids exerted their effects by acting on biological membranes (Mavromoustakos, Yang, & Makriyannis, 1995); however, this hypothesis was challenged following the discovery of an endogenous cannabinoid system along with endogenous agonists (mainly anandamide and 2-arachidonoyl glycerol, termed endocannabinoids) of the cannabinoid receptors (Pacher, Bátkai, & Kunos, 2006).

The two major cannabinoid receptors in the endocannabinoid system have been classified as Cannabinoid Receptor Type 1 (CB_1) and Cannabinoid Receptor Type 2 (CB_2) (Pertwee, 2008a). The CB_1 receptor is more responsive to psychoactive cannabinoids, such as THC, than to non-psychoactive cannabinoids, such as CBD (Matsuda, Lolait, Brownstein, Young, & Bonner, 1990). Interestingly, there is a high concentration of CB_1 receptors in the brain. The crystal structure of the human CB_1

receptor was elucidated by Hua et al. in 2016, providing not only a further understanding of how the endogenous ligands bind but also a rationale for CB_1 drug design.

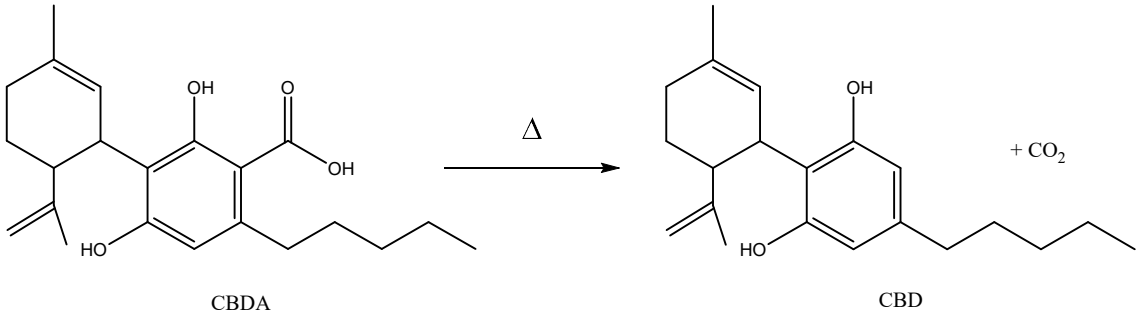
Although CB_2 receptors are found primarily in membranes of the immune system cells, such as those found in lymph tissues, they have been observed as being more highly expressed on various cancerous cells. Activation of G-coupled inhibitory proteins (G_i) associated with these cell membrane receptors leads to inhibition of adenylyl cyclase as well as many other signaling transduction pathways that modulate cell function (Alexander, Smith, & Rosengren, 2009). Cannabinoids, via these cannabinoid receptors, have the ability to regulate cellular signaling pathways which are critical for cell growth and survival. Therefore, cannabinoids may be useful in the treatment of cancer because of the high concentration of CB_2 receptors in tumor cell membranes (Alexander et al., 2009). With the emergence of more research on the endocannabinoid system and the role of cannabinoids as agonists in this system, the use of cannabinoids as therapeutic agents has become an attractive prospect.

Phytocannabinoids from *Cannabis sativa* have been reported to possess a variety of medicinal properties including antiproliferative activity in animal models toward specific solid tumors such as brain, pancreas, breast, prostate, colon, liver, and lung cancers (Massi et al., 2004). These antiproliferative properties are expressed when receptors of the endocannabinoid system are bound by specific phytocannabinoids. For example, CBD displays an unexpectedly high potency as an agonist of CB_1 and CB_2 in cells expressing these receptors; indeed, the manner which it interacts with CB_2 receptors provides a possible explanation for CBD's ability to interfere with immune cell chemotaxis and migration (Pertwee, 2008b).

Along with the ability of cannabinoids to inhibit tumor growth which has been observed *in vitro* and *in vivo* in several mice models, cannabinoids have been observed to demonstrate selective cellular toxicity, inducing cell death *in vitro* in human brain tumor cells at concentrations that are not harmful to normal cells (Salazar et al., 2009). This selective toxicity is significant because an effective anti-cancer treatment should induce apoptosis in tumor cells while not effecting healthy cells. Many cancer treatments in clinical use today do not possess this selective property and thus affect healthy cells producing unwanted side effects in contrast to cannabinoid-based treatments which potentially produce very few, if any, side effects (Robson, 2001). The potential for treatments with this selective nature with minimal side effects makes cannabinoids the interest in this study.

Currently, there are several commercial cannabinoid-based treatments available which utilize this cannabinoid activity in the body including Sativex®, Marinol®, and Epidiolex® (GW Pharmaceuticals, 2018). Sativex® is a THC-CBD-enriched, botanical extract for treating moderate to severe symptoms of multiple sclerosis including pain and spasm and was the first drug to have been approved by the FDA with two active ingredients in a single drug. Marinol®, a synthetic THC analog, alleviates nausea and emesis in cancer patients undergoing chemotherapy and has also been demonstrated to mitigate anorexia and cachexia associated with AIDS or chemotherapy. The most current drug approved by the FDA is the CBD-based drug Epidiolex® used to treat seizures associated with Dravet syndrome and tuberous sclerosis (GW Pharmaceuticals, 2018).

In consideration of growing support for cannabinoids as safe, effective drugs currently available for epilepsy, as well as research supporting cannabinoids as candidates for targeted anti-cancer therapy, an efficient delivery system needs to be developed to address any obstacles which may be present when proposing a cannabinoid-based drug. First, cannabinoids are synthesized in plants as the acidic form CBDA or THCA, for example, which serve as precursors to the therapeutically-active forms CBD and THC, respectively. To convert the physiologically-inactive acidic form from the plant (termed the prodrug) to an active drug, the cannabinoid-based component must be decarboxylated to remove its acidic functional group (Figure 2).



CBDA CBD

Figure 2: Decarboxylation of cannabidiolic acid (CBDA) to cannabidiol (CBD).

A simple way to decarboxylate these compounds is to heat them, usually accomplished by smoking or baking *Cannabis* plant material. However, this is not an ideal way to deliver a proper dose of the active drug to the site of the tumor. Active drugs comprise only a small portion of the *Cannabis sativa* plant; thus when absorbed through ingestion or inhalation most of the active drug will be distributed throughout the blood stream and only a small amount of therapeutic agent will actually reach the site of the tumor (Casarett & Doull, 2013). Additionally, if delivered in this manner one would need to consider the psychoactive properties of Δ^9-THC, the concentration of which

varies from each strain of the plant, and how it would affect adult and child patients regarding cognitive function, reaction time, learning and memory functions.

Enzymatic decarboxylation of cannabinoids was explored in this study as an alternative method to heating. While humans do not possess the enzyme(s) necessary to catalyze the decarboxylation of cannabinoid compounds, several cell types, both prokaryotic and eukaryotic, have been reported to possess enzymes capable of catalyzing this specific reaction using substrates with functional groups similar to the cannabinoids (Grant & Patel, 1969; Santha, Rao, & Vaidyanathan, 1996; Kirimura, Gunji, Wakayama, Hattori, & Ishii, 2010; Snini et al*,* 2013). Many of these cell lines reported in the literature were explored in this study to identify an organism with the appropriate enzyme capable of this decarboxylase activity. Those organisms which exhibit the most potential will be considered for gene isolation of the target enzyme which could then be transfected into a eukaryotic cell of choice. The modified cell would then be encapsulated using Cell-in-a-Box® cellulose-based live cell encapsulation technology (PharmaCyte, 2017) and injected directly upstream from a tumor. This would allow for the appropriate dosage of a highly-targeted therapeutic drug to be converted directly to the active drug at the site of the tumor, making it an attractive cancer treatment.

To identify an enzyme that could activate the naturally occurring prodrug CBDA to its active drug form CBD, four different bacterial species and three fungal species were screened for activity; these species included *Enterobacter (Klebsiella) aerogenes, Pseudomonas putida, Klebsiella pneumonia, Bacillus subtilis, Aspergillus clavatus, Aspergillus niger,* and *Penicillium chrysogenum.* The microbial cells, both intact and lysed, were incubated with the cannabinoid prodrug. The cannabinoid substrate and

product(s) were simultaneously extracted from the incubated cell suspension at appropriate time intervals during incubation and analyzed using high-performance liquid chromatography (HPLC). The retention times of peaks in the chromatograms were compared to known standards to determine if the prodrug was activated by the species assessed.

In this study, identification and quantification of cannabidiol was assessed through the use of HPLC. The most common methods for qualitative and quantitative analysis of phytocannabinoids from *Cannabis sativa* include GC/FID and GC/MS (Klein, 2015); however, although these methods are rapid and well established, these methods are not useful for heat-labile compounds which can be decarboxylated via heat. Instead, an analytical HPLC method for monitoring conversion of CBDA to CBD and a preparatory HPLC method for isolation of CBDA from crude *Cannabis* extract were developed. This allowed for the initial steps of development of an enzyme-driven, cannabinoid-based drug to be performed in-house, from plant to active drug.

CHAPTER II

REVIEW OF LITERATURE

Cannabinoids can be classified into three major categories; endocannabinoids, phytocannabinoids, and synthetic cannabinoids (Pertwee, 2008a). Endogenous endocannabinoids are made in animals, including humans; in contrast, phytocannabinoids are plant-derived products from *Cannabis* while synthetic cannabinoids are laboratory produced. Although a brief mention of endocannabinoids is presented, this review focuses on the structure, function, and biological activity of phytocannabinoids. In addition, a broad review of many different *in vitro* and *in vivo* studies on various cancer cell lines is presented as a rationale for cannabidiol as a targeted cancer chemotherapeutic agent. Enzymatic conversion of cannabinoid model compounds is also discussed in support of an enzyme-based conversion of the inactive prodrug cannabidiolic acid to cannabidiol. Finally, Cell-in-a-Box® technology is discussed as a potential mechanism of delivery using a genetically-engineered cell containing the gene necessary for an enzyme capable of catalyzing the conversion of prodrug to active drug.

Cannabinoids

Phytocannabinoids from *Cannabis sativa* are synthesized in their acidic forms, from isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the precursors of all terpenoids (Fellermeier, Eisenreich, Bacher, & Zenk, 2001). Although believed to be biosynthesized via the mevalonate pathway prior to the 1990s, it has been

suggested that the precursors IPP and DMAPP are derived predominantly via the deoxyxylulose phosphate pathway (Eisenreich, Rohdich, & Bacher, 2001). An overview of the biosynthesis of major cannabinoids from these precursors is shown in Figure 3. In general, IPP (1) and DMAPP (2) are condensed to form geranyl diphosphate (3) which then combines with olivetolic acid (4) to form cannabigerolic acid (5) from which the remaining cannabinoids are derived. Due to their origin from these two isoprene derivatives and their phenolic properties, cannabinoids can be categorized as terpenophenols.

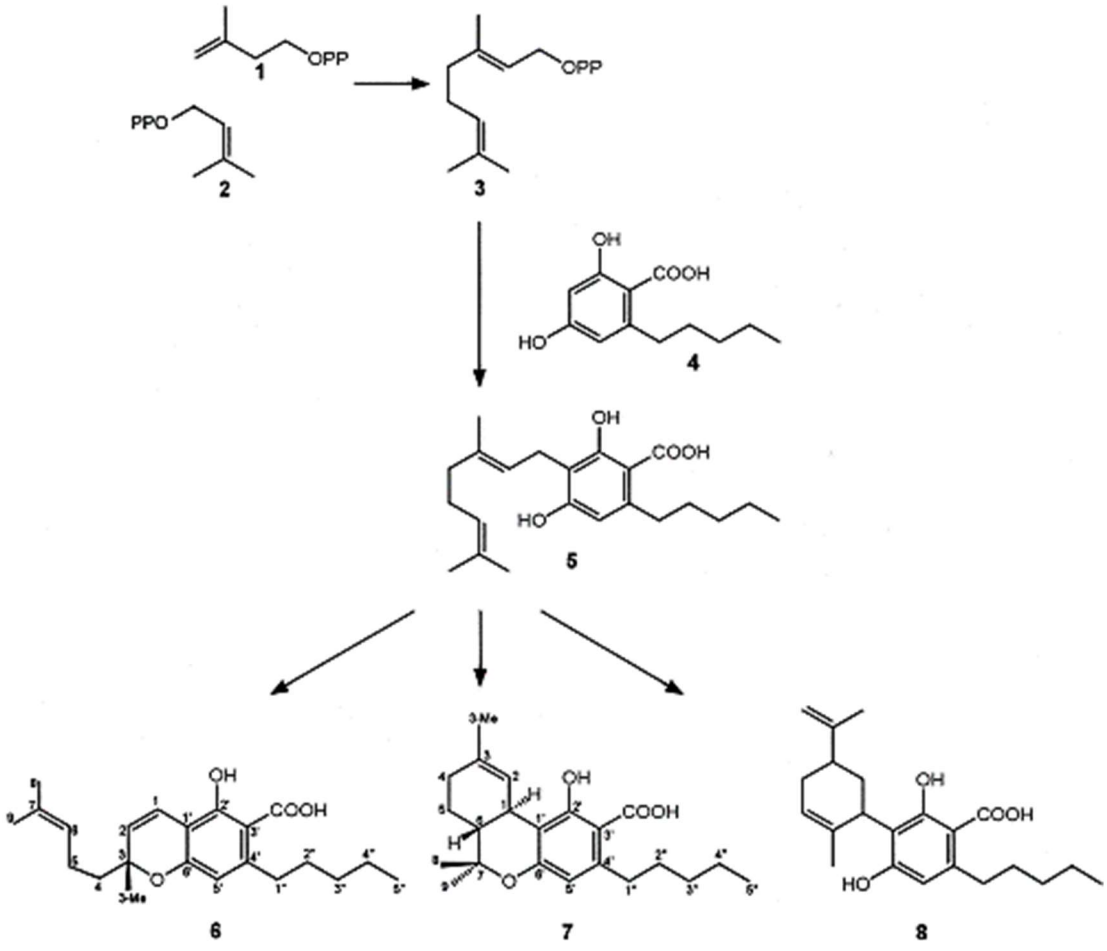


Figure 3: Biosynthesis of cannabichromenic acid (6), tetrahydrocannabinolic acid (7), and cannabidiolic acid (8) from the precursors IPP (1) and DMAPP (2). The intermediates geranyl diphosphate (3), olivetolic acid (4), and cannnabigerolic acid (5) are also shown. From Fellermeier et al. (2001), used with permission (Appendix C).

Currently, over 150 cannabinoids have been characterized from *Cannabis sativa* (ElSohly & Gul, 2016). These cannabinoids can be categorized based on structural similarities as tetrahydrocannabinols (THCs), cannabidiols (CBDs), cannabinols (CBNs), cannabigerols (CBGs), cannabichromenes (CBCs), and cannabinodiols (CBNDs), the most abundant of which are THCs, CDBs, and CBNs. Those which are deemed structurally unique cannabinoids are categorized as miscellaneous (ElSohly & Slade, 2005). Structures of representative compounds from each category are shown in Figure 4.

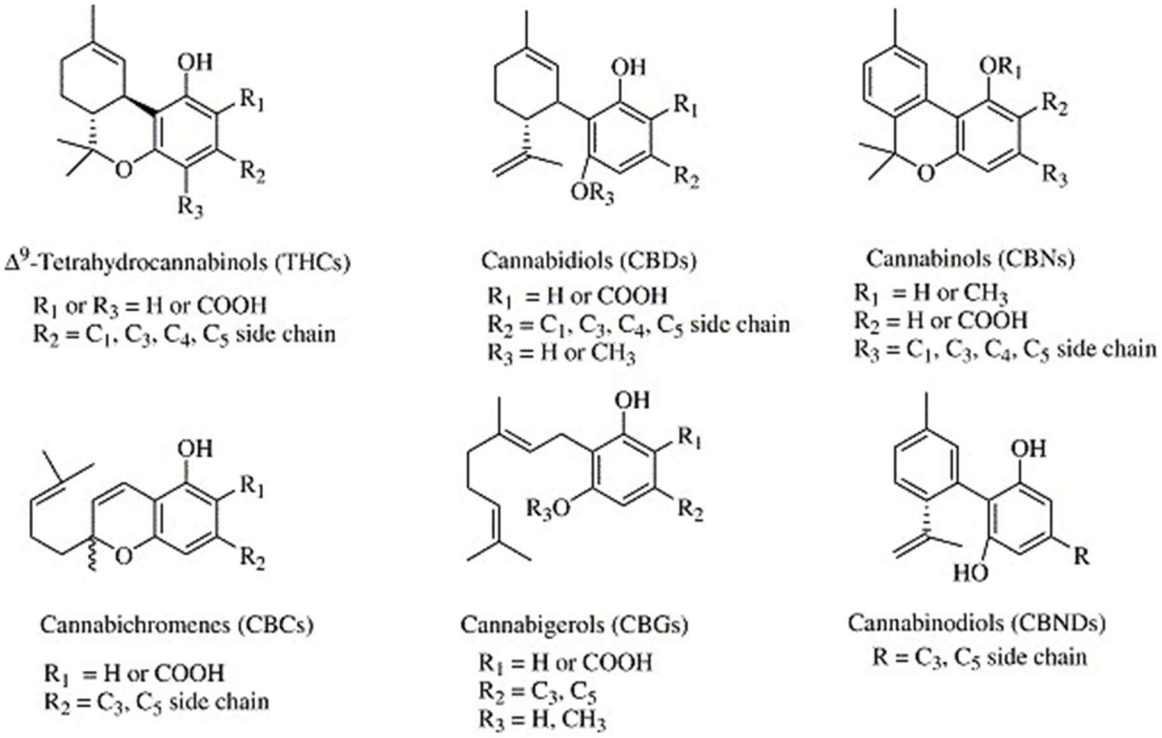
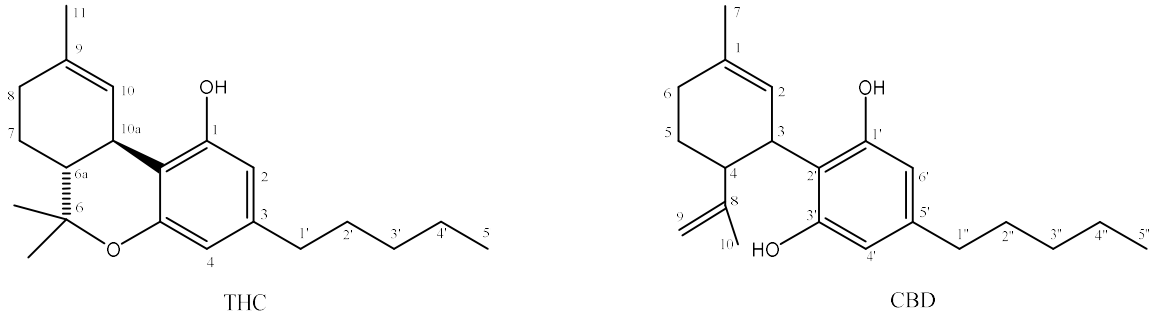


Figure 4: Representative structures of THCs, CBDs, CBNs, CBGs, CBCs, and CBNDs. From Thakur, Duclos, & Makriyannis (2005), used with permission (Appendix C).

Although there is much structural variance among these six major categories of cannabinoids, most are composed of 21 carbons which includes an n-pentyl chain on C-3 (THCs) or 5'-C (CBDs) of the aromatic ring. The variability of functional group

moieties accounts for the difference in physiological activity among cannabinoids (Thakur et al., 2005). This variability in structure also causes chemical nomenclature of certain cannabinoids to differ, for example the numbering of CBD compared to THC. These two structures are closely related, and CBD has been shown to cyclize into THC under acidic conditions (Gaoni & Mechoulam, 1964); however, due to their differences in functionality, they are numbered differently. Numbering for THC is therefore determined by a pyran ring; CBD relies on a terpene ring for numbering priority (Mechoulam & Hanuš, 2002). These numbering systems are given in Figure 5.



THC CBD

Figure 5: Numbering system for THC and CBD.

The remainder of this review will focus predominately on the related cannabinoids CBDA and CBD. CBD is distinguished by its aromatic and terpene rings, which lay nearly perpendicular to each other. Two independent forms, which differ mainly in the conformation of the pentyl side chain, were noted by Jones et al. (1977) when elucidating the crystal structure. The major torsion-angle difference is for the C (5')-C(1'')-C(2'')-C(3''), which is -57.2 and 179.2° for forms 1 and 2, respectively. Also, the benzene ring in the first form is planar and all substituents are coplanar while in the second form the aromatic ring is twisted slightly with a torsion angle of -4.1° and the 3'-OH bent slightly out of the plane of the ring. CBDA differs from CBD by a

carboxylic acid group on the 6' carbon *ortho* to the phenolic hydroxyl (Mechoulam & Hanuš, 2002). The decarboxylation of CBDA allows it to act as a prodrug where, upon decarboxylation, different biological effects can be noted (Ligresti, 2006).

Cannabinoid Physiology

The ability of cannabinoids to regulate the endocannabinoid system and the role of its receptors has been supported in numerous studies. Shortly after the characterizations of several phytocannabinoids, starting with the characterization of Δ^9-THC by Raphael Mechoulam in the 1960s, researchers began to speculate how cannabinoids exert their effects in the body. Although it was originally assumed that cannabinoids exerted their effects by acting on biological membranes due to their lipophilic nature (Mavromoustakos et al., 1995), it was later discovered that the biological effects exerted by cannabinoids is facilitated through binding to endogenous cannabinoid-like receptors (Fonseca, Costa, Almada, Correia-Da-Silva, & Teixeira, 2013). This is not to say that cannabinoids only act upon the endogenous endocannabinoid system, merely that it is the primary mode of action.

Two major cannabinoid receptors of the endocannabinoid system have been identified as Cannabinoid Receptor Type 1 (CB_1) and Cannabinoid Receptor Type 2 (CB_2) (Pertwee, 2008a). The crystal structure of the human CB_1 receptor was elucidated by Hua et al. in 2016. The characterization of these receptors allowed for a more in-depth understating of ligand-receptor interactions and helps provide rationale for drug discovery. CB_1 receptors are highly expressed in nociceptive areas in the brain, the cerebellum, limbic system, and basal ganglia. This location of the CB_1 receptor makes it more responsive to psychoactive cannabinoids, such as THC, than to non-psychoactive

cannabinoids, such as CBD (Matsuda et al., 1990). Those receptors located in the brain are sparse in density in lower brainstem areas controlling cardiovascular and respiratory function, which may explain why high doses of cannabinoids are not lethal (Herkenham et al., 1990). CB_1 receptors have also been detected in spleen, eye, uterus, and testes (Guzmán, 2003).

In contrast, the CB_2 receptors are found mainly in peripheral tissues and tissues of the lymph nodes, tonsils, and spleen. Due to these locations, CB_2 is mainly a peripheral immunomodulation receptor with an important role in mediating pain, inflammation, and physiological defense (Pacher et al., 2006). The CB_2 receptor has also been observed in tumor cells. Both CB_1 and CB_2 receptors belong to the G-protein-coupled family of receptors and thus are mediated by activation of $G_{i/o}$ alpha subunits (Turu & Hunyady, 2010). Activation of these G-coupled cell-membrane proteins may lead to inhibition of adenylyl cyclase as well as many other signaling transduction pathways that modulate cell function (Alexander, Smith, & Rosengren, 2009).

Shortly after the identification of the CB_1 and CB_2 receptors, research expanded from just studying cannabinoids in marijuana to looking for cannabinoid-like compounds produced in the body that act on the endogenous cannabinoid receptors. The endogenous agonists anandamide and 2-arachidonoyl glycerol were discovered, revealing that the primary physiological function of the endocannabinoid system was to bind these endogenous ligands (Pacher, Bátkai, & Kunos, 2006). These endogenous ligands (endocannabinoids) are made within the cell and are synthesized on-demand throughout the body from precursors in lipid membranes (Alger, 2013), so they can be used exactly when and where they are needed in the body, rather than being packaged and stored

similar to other signaling molecules. Endocannabinoids travel in a retrograde fashion to inhibit neurotransmitter release, making the main function in the brain to attenuate release of neurotransmitters (Guzmán, 2003). These receptors, along with the endocannabinoids themselves and their regulatory enzymes, make up the endocannabinoid system which is present in many chordates of the animal kingdom (Elphick, 2012). Activation of this system is associated with various processes including regulation of appetite, motor activity, memory, learning, emesis, and nociception (Fernández-Ruiz et al., 2007). More generally, it can be said that the role of the endocannabinoid system which has been observed is a role in homeostasis by controlling metabolic functions such as energy storage and transport and immune function (Pagotto, Marsicano, Cota, Lutz, & Pasquali, 2006).

In a 1998 report by Marzo et al., the functions of endocannabinoids were summarized as aids to "relax, eat, sleep, forget, and protect" with a focus on the endocannabinoids and their relation to the sensation of hunger. The amount of leptin, a hunger-inhibiting molecule, was shown to be inversely correlated with the number of endocannabinoids produced suggesting a relationship between the endocannabinoid system and appetite. If the endocannabinoid system is stimulated (by endo- or phytocannabinoids), an individual will have less leptin, and therefore will feel hungrier. These results interestingly align with anecdotal evidence from recreational *Cannabis* users who self-reported feeling hungrier when ingesting more and more *Cannabis*.

Unsurprisingly because of the large number of cannabinoid receptors in the brain, the endocannabinoid system is also reported to be involved in the regulation of memory (Alvares, Genro, Diehl, & Quillfeldt, 2008). A common effect self-reported by *Cannabis*

users is short-term memory impairment. In 2009, Mazzola et al. reported a significant impairment in the memory of rats when Δ^9-THC was injected intra-peritoneally at concentrations of 3.0, 5.0, and 6.0 mg/kg 30 minutes prior to a passive-avoidance task. Long term potentiation in the hippocampus, a process essential for formation of storage of long-term memory, has been shown to be suppressed in mice treated with THC (Hampson & Deadwyler, 1999). The role of the endocannabinoid system in memory is also supported by the observation that CB_1 knockout mice show enhanced memory and long-term potentiation (Jiang, 2005). Although it is generally agreed upon that cannabinoids do have a regulatory role in memory, contradictory information on whether these effects impair or improve different phases of the memory processes exist simultaneously. Impairments in object recognition have been linked to dysregulation of the glutamatergic system (Nilsson, Hansson, Carlsson, & Carlsson, 2007) as well as hippocampal and para hippocampal dysfunction (Fernández-Ruiz et al., 2007). Interestingly, a study found that CBD interacts with the glutamatergic system (Hallak et al., 2011); CBD augmented the effects of an N-methyl-d-aspartate (NMDA) receptor antagonist in a human study, a receptor which is associated with memory and learning impairments. CBD was also shown to be protective against glutamate neurotoxicity (Hampson & Deadwyler, 1999). Finally, memantine, another NMDA receptor antagonist, improved object recognition in a transgenic Alzheimer Disease mouse model (Scholtzova et al., 2008) suggesting that CBD may improve recognition memory via the glutamatergic pathway. Although much has been learned about the endocannabinoid system, as of yet, it is still incompletely understood.

Cannabidiol and Cancer

This thesis research is grounded in much evidence from prior research into the effectiveness of cannabinoids, and more specifically cannabidiol, as cancer treatments. Cannabidiol is of specific interest as a cancer drug because it has many of the same antiproliferative effects as other cannabinoids without the psychotropic effects (Izzo, Borrelli, Capasso, Di Marzo, & Mechoulam, 2009). Cannabinoids could be useful in the treatment of cancer due to their ability to regulate cellular signaling pathways critical for cell growth and survival (Alexander et al., 2009). Expression of CB_1 and CB_2 receptors has been found in many types of cancer cells, although this does not necessarily correlate with the expression of these receptors in the tissue of origin (Guzmán et al., 2006). The fact that these receptors are upregulated in cancerous tissue and not in the surrounding tissue may explain the observation by McAllister et al. (2005) that cannabinoids can selectively inhibit proliferation and induce cancerous cell death at concentrations that do not harm normal cells.

The expression of CB_1 and CB_2 receptors in tumors of patients with hepatocellular carcinoma was studied by Xu et al. (2006). Patients were treated with embolization therapy to essentially starve the cancerous tumors of blood flow and essential nutrients. Patients then underwent a hepatectomy and liver samples were preserved for analysis. Their study concluded that when higher numbers of CB_1 and CB_2 receptors are expressed in cancerous tissue, there is a correlated improvement in prognosis in patients with hepatocellular carcinoma (Xu et al., 2006) and potentially many other cancerous cell lines. Therefore, cannabinoids may be useful in the treatment of cancer because of the high concentration of cannabinoid receptors in tumor cells (Alexander et al., 2009) which

are selectively targeted by cannabinoids. Studies on the effect of CBD on prostate, breast, glioma, and other cancer cell lines are reviewed later in this section.

In general, activation of CB_1 by cannabinoids minimizes membrane depolarization on presynaptic cells by inhibiting voltage-gated Ca^{2+} channels and activation of voltage-gated K^+ channels (Turu & Hunyady, 2010). The result is a decrease in neurotransmitter release from presynaptic cells. Activation of the cannabinoid receptors in cancer cells has been reported to increase the levels of ceramide which has been shown to activate a mitogen-activated protein kinase (MAPK) pathway known as the extracellular-signal-regulated kinase (ERK) survival pathway (Ellert-Miklaszewska, Grajkowska, Gabrusiewicz, Kaminska, & Konarska, 2007; Ozaita, Puighermanal, & Maldonado, 2007) . Activation of the ERK pathway usually results in cell proliferation, however continual stimulation causes arrest of the cell cycle and apoptosis. Cannabinoids have also been shown to exert anti-proliferative effects on cancer cells by causing an arrest of the cell cycle through the inhibition of adenylyl cyclase (Fonseca et al., 2013). This pathway can occur through activation of either CB_1 or CB_2 since they are both coupled to inhibitory G-proteins. Inhibition of adenylyl cyclase leads to lower concentrations of intracellular cAMP thus decreasing the activity of protein kinase A (PKA) and also activating the ERK pathway similar to the effect by increased levels of ceramide (Figure 6).

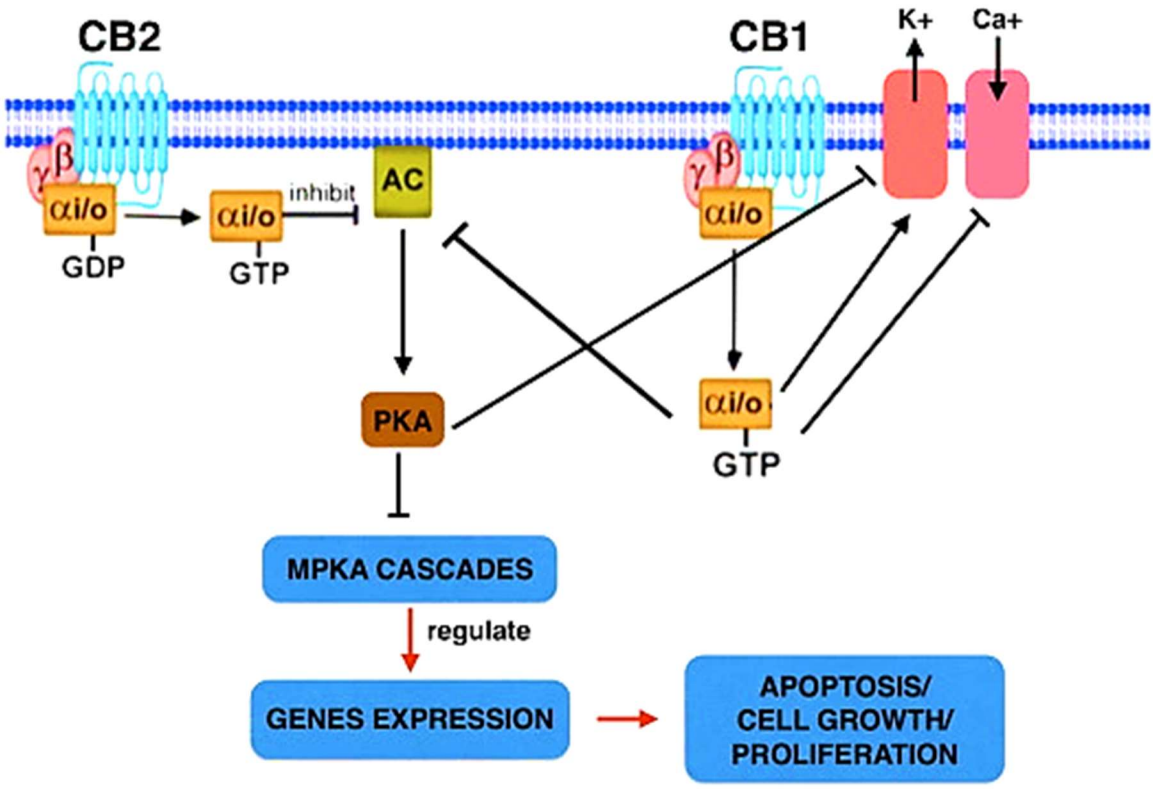


Figure 6: Schematic of CB_1 and CB_2 cellular signaling. From Katchan, David, and Shoenfeld (2016), used with permission (Appendix C).

Other potential pathways include transient receptor potential cation channel, subfamily V, member 1 (TRPV1) activation, downregulation of the vascular endothelial growth factor (VEGF) pathway, production of reactive oxygen species (ROS), induction of endoplasmic reticulum (ER) stress, and the activation of both intrinsic and extrinsic apoptotic pathways (Ramer, Merkord, Rohde, & Hinz, 2012). The mechanism of tumor cell inhibition produced by CBD was shown to depend on the type of cancer cell being analyzed (Massi, Solinas, Cinquina, & Parolaro, 2013). Importantly, of the cannabinoids CBD seemed to involve direct TRPV1 activation and/or CB_2 indirect activation (via fatty acid amide hydrolase), as well as induction of oxidative stress (Ligresti, 2006). Due to its many mechanisms of action, some of which are noted in Figure 7, cannabidiol is of interest in treating a variety of cancers. Activation of CB receptors can be responsible for

the increase in levels of ceramide or decrease of cAMP level via inhibition of adenylyl cyclase, both events lead to apoptosis mediated by caspase activities. Also, the ability for TRPV receptors activated by cannabinoids to cause an increase in the level of ROS and oxidative stress which leads to apoptosis has been observed. Although each mechanism may be activated differently, the process is highly interconnected and each event will lead to the same end effect, tumor cell death.

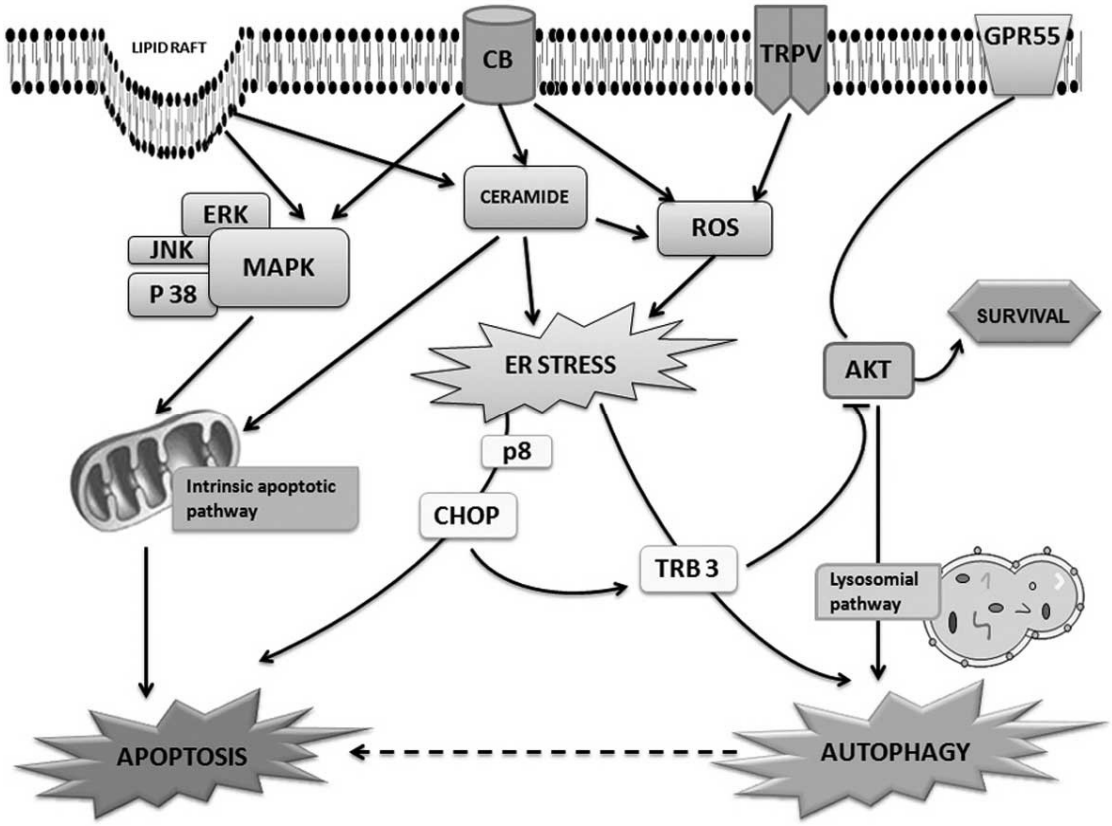


Figure 7: Schematic of various cell-death pathways potentially regulated by CBD. From Calvaruso, Pellerito, Notaro, & Giuliano (2012), used with permission (Appendix C)

Prostate cancer is the most common non-skin cancer among men in the United States (National Cancer Institute, 2017). The *in vitro* antitumor and anti-inflammatory activities of CBD in human prostate cancer cell lines were investigated by Sharma, Hudson, Adomat, Guns, & Cox (2014). Treatment of human prostate cancer cell lines

LNCaP and PC3 with *Cannabis* extracts containing 20–60 µg/mL CBD showed negligible activity at the lowest concentration (20 µg/mL) and massive induction of apoptosis at the highest concentration (60 µg/mL). This supports the need for a targeted approach to delivery and precision dosing when using CBD as a therapeutic drug.

Also examined in the Sharma et al. (2014) study was the effect of a *Cannabis* extract on androgen receptor transcripts. Because over-expression of androgen receptors in prostate cancer cells may promote cell growth, inhibiting this expression with the use of CBD may prove beneficial in preventing tumor growth. Treatment of LNCaP cells with *Cannabis* extract prepared in dimethyl sulfoxide (DMSO) containing 20, 40, 60, and 70 µg/mL CBD resulted in a marked decrease in androgen receptor expression when assessed at 24 hours post treatment. Although a significant decrease in expression was observed at all concentrations of CBD, the highest concentration produced the largest decrease in androgen receptor expression.

Additional studies of human prostate cancer cells *in vitro* and *in vivo* using mouse models found that the CB_2 receptor was involved in the inhibition of prostate cancer cell growth in both experimental models (Olea-Herrero, Vara, Malagarie-Cazenave, & Díaz-Laviada, 2009). Petrocellis et al. (2012) studied the effects of several pure cannabinoids (CBC, CBD, CBG, CBN, CBDA, CBGA, CBDV (cannabidivarin), CBGV (cannabigevarin), THC, THCA, THCV (Δ^9-tetrahydrocannabivarin), THCVA (Δ^9-tetrahydrocannabivarin acid)) and those in *Cannabis* extracts on different human prostate carcinoma cell lines both *in vitro* and *in vivo*. Of the pure cannabinoids studied, CBD (1–10 µM) was the most effective inhibitor of tumor growth *in vitro*. *In vivo* studies were done with a *Cannabis* extract which contained 64.4% (w/w) CBD since it proved to be

even more potent than pure CBD alone when evaluating cell viability via an MTT assay in another portion of the study. Indeed, when the extract was administered to tumors generated in athymic mice from LNCaP cells, the growth of these tumors was reduced (Petrocellis et al., 2012).

Breast cancer is the most common cancer in women, regardless of race or ethnicity, in the United States (National Cancer Institute, 2017). Several cannabinoids were screened for their ability to reduce cell proliferation of different breast tumor cell lines (MCF7 and MDA-MB-231) *in vitro* and *in vivo* in a mouse model by Ligresti (2006). CBD exhibited the highest potency of cannabinoids studied, with IC_{50} values being 8.2 ± 0.3 μM (MCF7) and 10.6 ± 1.8 μM (MDA-MB-231). CBD selectively inhibited the growth of these two breast cancer cell lines *in vitro* at a range of concentrations (2.0-25 μM). Cannabidiol at a concentration similar to its IC_{50} did not affect the vitality of nontumor cell lines. Only at a concentration as high as 25 μM, which inhibited 100% of tumor cell growth, was viability of non-cancerous cells affected. Data regarding the extent to which the healthy cells were affected were not noted in the study. Mice with xenograft tumors obtained by subcutaneous injection of human MCF7 and MDA-MB-231 breast carcinoma cells were treated *in vivo* with either pure cannabidiol (5 mg/kg) or a cannabidiol-rich extract (6.5 mg/kg) and exhibited significantly smaller tumors in comparison with control mice.

In addition to confirming the *in vitro* observation by Ligresti (2006) of a reduction in proliferation of the human breast cancer cell line MDA-MB-231 when exposed to CBD, McAllister et al. (2007) reported a reduction in cancer cell proliferation in an additional breast cancer cell line, MDA-MB-436. Cells were treated with 0.1, 1.0 or 1.5

μM solutions of CBD for three days. Because the 1.5 μM solution was the most antiproliferative, the McAllister group examined the inhibitory effect of CBD at this concentration on Id-1 expression. Id-1, an inhibitor protein of basic helix-loop-helix transcription factors, has been shown to be a key regulator of the metastatic potential of breast and additional cancers (Benezra, Davis, Lockshon, Turner, & Weintraub, 1990). McAllister et al. (2005) previously demonstrated that reducing Id-1 led to significant reductions in breast cancer cell proliferation *in vitro* suggesting that reducing Id-1 expression could be a therapeutic strategy. Their 2007 study found that after a three-day treatment with CBD (1.5 μM, daily), the mRNA transcription of the Id-1 gene was significantly inhibited. Minn et al. (2005) had previously shown that CBD regulated the expression of key genes involved in the control of cell proliferation and invasion via the down-regulation of Id-1. This down-regulation may be one of the mechanisms by which CBD can reduce tumor aggressiveness. More recently, it was discovered that the Id-1 down-regulation was mediated by the up-regulation of extracellular-signal-related kinase (ERK) phosphorylation which produces the active isoform of the ERK protein (McAllister et al., 2012). Previously mentioned studies already showed that over stimulation of the ERK pathway contributed to arrest of the cell cycle and apoptosis, further supporting the inhibitory action of CBD through the ERK pathway.

An *in vitro* study which demonstrated the concentration-dependent nature of CBD as an effective breast cancer treatment was reported by Shrivastava, Kuzontkoski, Groopman, & Prasad (2011). These researchers found that CBD induced apoptosis in human breast cancer cell lines MDA-MB-231, MCF-7, SK-BR-3, and ZR-75-1 in a concentration-dependent manner (Figure 8). Moreover, it was found that at these

concentrations, CBD had little effect on MCF-10A cells, a non-tumorigenic human mammary cell line. These studies support the desirability of CBD as an anticancer agent by demonstrating a selective toxicity for breast cancer cells.

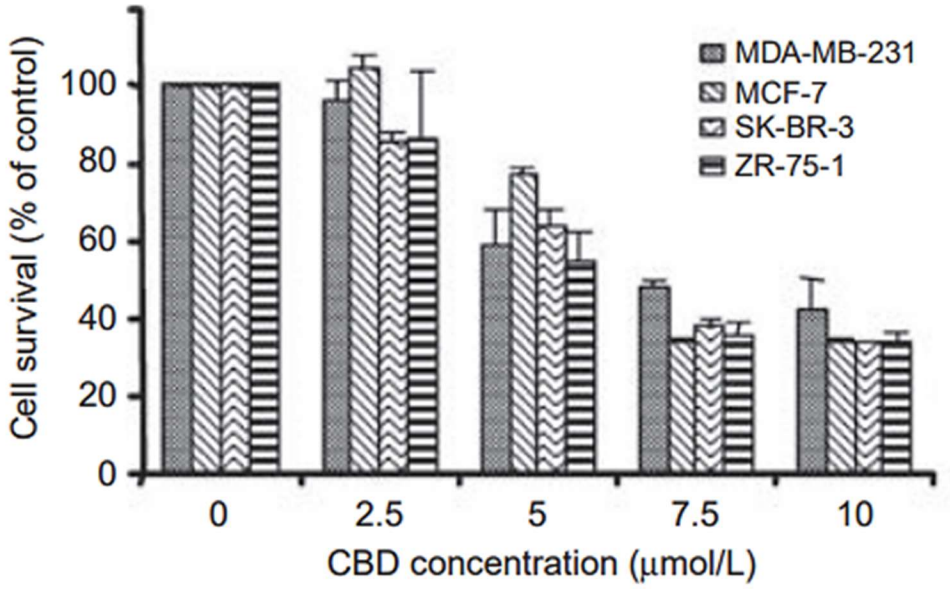


Figure 8: Cell survival (as measured by an MTT assay) of various breast cancer cell lines treated with increasing concentrations of CBD for 24 hours. From Shrivastava et al. (2011), used with permission (Appendix C).

Gliomas, or tumors of glial origin, are notably aggressive forms of cancer to which traditional forms of treatment such as radiation and chemotherapy are generally unresponsive (Philip-Ephraim, Eyong, Williams, & Ephraim, 2012). In a study by Massi et al. (2004), CBD (25 µM) was demonstrated to be effective in inhibiting U87-MG and U373 human glioma cell proliferation *in vitro* while not affecting healthy glial cells. *In vivo* treatment with CBD significantly reduced tumor growth of subcutaneously implanted U87 human glioma cells in mice when administered at a dose of 0.5 mg per mouse. In their study, it appeared that the addition of CBD to cell culture media during *in vitro* studies led to a dramatic drop of mitochondrial oxidative metabolism and

viability in the glioma cells. When adding the antioxidant α-tocopherol at a concentration of 10 μM the antiproliferative effect of CBD was significantly prevented. Inhibitory effects were observed more rapidly at concentrations close to their IC_{50} values with effects from CBD (25 μM) observed at 24 hours as compared to THC (1 μM) observed within 2-5 days as reported by Sanchez et al. (1998)

In a study by Cianchi et al. (2008), the cytotoxic effect of N-cyclopentyl-7-methyl-1-(2-morpholin-4-ylethyl)-1,8-naphthyridin-4(1H)-on-3-carboxamide (CB13), a synthetic CB_2 agonist, was investigated *in vitro* using the four colon cancer cell lines HCT8, SW480, HCA7, and HCT15 which had previously been reported to express the CB_2 receptor. The results of the study showed the growth of all cell lines was inhibited in a concentration-dependent manner. The CB_2 agonist also reduced growth of colon cancer cells *in vivo* in a mouse model (Cianchi et al., 2008). Additionally, an extract of *Cannabis sativa* with a high content of CBD was demonstrated to reduce proliferation in colorectal cancer cells but not in healthy cells in a study by Romano et al. (2013). Proliferation was evaluated by an MTT assay in both healthy and cancerous cells at concentrations of CBD ranging from 0.3-5 μM CBD for 24 hours. Twenty percent inhibition of tumor cell growth was reported with the highest concentration of CBD.

An *in vivo* study using athymic nude mice revealed a significant inhibition of A549 lung tumor metastasis in animals treated with a dose of 5 mg/kg CBD every 72 hours for 28 days as compared to vehicle-treated controls. The number of metastatic lesions in mice treated with CBD was one nodule after 28 days while the number of nodules in mice treated with only the vehicle was six, yielding an 84% inhibition of metastasis (Ramer et al., 2012).

Enzymatic Decarboxylation

The carboxylic acid group *ortho* to the hydroxyl group on the aromatic ring of CBDA must first be removed to elicit the desired physiological effects (Ligresti, 2006*)*. Many studies have reported the expression of an enzyme capable of catalyzing the decarboxylation of phenolic carboxylic acids and other structurally similar acids by several eukaryotic and prokaryotic species which include many from the genera *Pseudomonas, Klebsiella, Bacillus, Aspergillus,* and *Penicillium* . In order to prioritize substrates based on similarity to CBDA, they were organized into four categories with Category 1 being the most similar and Category 4 being the least similar to the phenolic carboxylic acid moiety of CBDA. These compounds were of interest because of their similarity of the aromatic ring moiety, which interacts with the catalytic domain of these decarboxylase enzymes, to the aromatic moiety of CBDA. For proper enzyme-substrate binding to occur, there must be similarity between compounds so that important regions of the binding site can interact properly with the substrate in order for the desired reaction to occur.

Substrates such as salicylic acid, 6-methylsalicylic acid, 2,3-dihydroxybenzoic acid and gentisic acid (2,5-dihydroxybenzoic acid) have hydroxyl groups *ortho* to the carboxyl group, making them similar in structure to CBDA. Structures of these Category 1 substrates are presented in Figure 9. Cell lines capable of catalyzing decarboxylation of these Category 1 substrates are reported below.

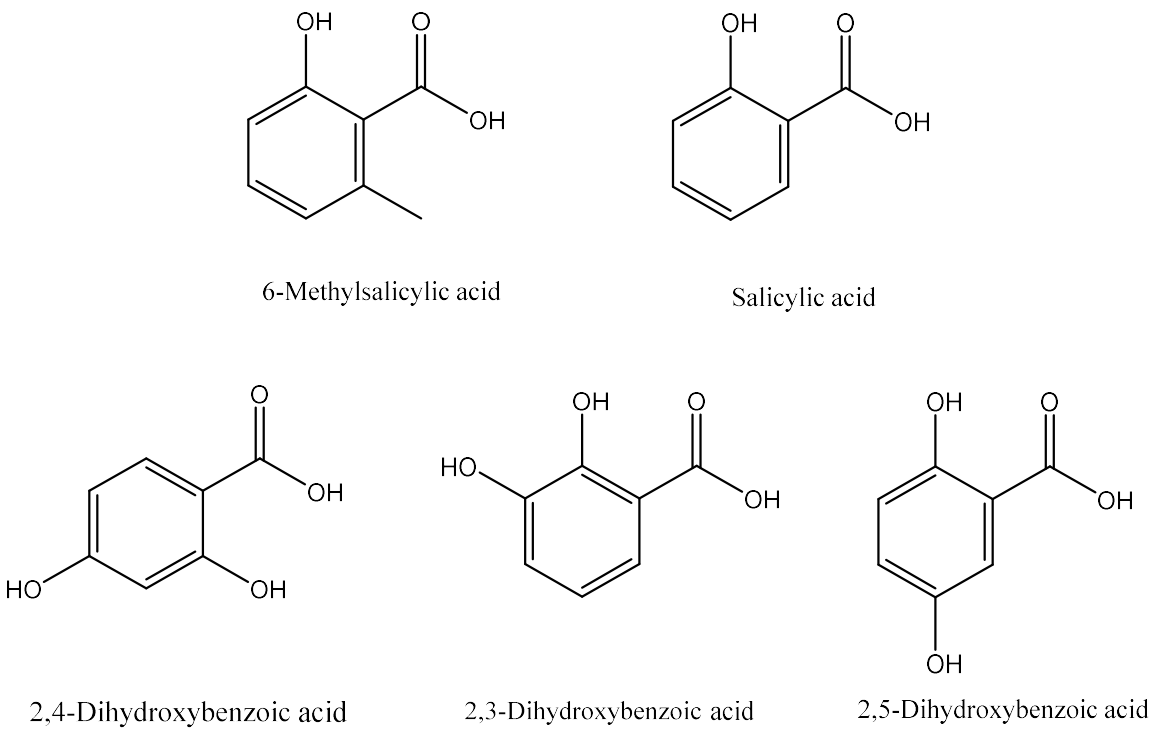


Figure 9: Structures of Category 1 substrates.

Bacillus subtilis will express *padC* and *bsdBCD* genes when induced with salicylic acid (Duy et al., 2007). *PadC* encodes for a phenolic acid decarboxylase (PAD) while the *bsdBCD* operon encodes for phenylacrylic acid decarboxylase B and 4-hydroxybenzoate decarboxylase C and D (Duy et al., 2007). Salicylic acid, a Category 1 substrate, was unsurprisingly decarboxylated to phenol by the phenolic acid decarboxylase from *padC* but also was decarboxylated by the phenylacrylic acid decarboxylase encoded by *bsdBCD* even though salicylic acid is not, in fact, a phenylacrylic acid (Duy et al., 2007). However, it is known that genes for these enzymes are down-regulated when stress is absent (Duy et al., 2007) and so induction was necessary.

Trichosporon moniliform was shown to produce the enzyme salicylic acid decarboxylase, which will catalyze the decarboxylation of not only salicylic acid but

other carboxylic acids as well (Kirimura et al., 2010). The enzyme also catalyzed the decarboxylation of b-resorcylic acid (2,4-dihydroxybenzoic acid) producing resorcinol (1,3-dihydroxybenzene), c-resorcylic acid (2,6-dihydroxybenzoic acid) producing resorcinol, 2,3-dihydroxybenzoic acid producing catechol (1,2-dihydroxybenzene), and 4-aminosalicylic acid producing 3-aminophenol.

Multiple fungal representatives have been reported to express a variety of decarboxylases. A well-classified enzyme transiently expressed in *Aspergillus* recognizes 6-methylsalicylic, a substrate containing a carboxylic acid *ortho* to a phenolic hydroxyl and a carbon side chain on C-6 which would be analogous to the pentyl chain on C-5' of CBD. The gene *PatG,* encoding this enzyme, is expressed by many fungi including *Aspergillus clavatus, Aspergillus giganteus,* and *Aspergillus longivesica* as well as *Byssochlamys nivea,* and *Penicillium expansum* (Snini et al., 2013).

The non-oxidative decarboxylation of 2,3-dihydroxybenzoic acid (2,3-dHBA) to catechol is catalyzed by the enzyme 2,3-dihydroxybenzoic acid decarboxylase (2,3-dHBAD). This enzyme is produced by several *Aspergillus* fungi including *Aspergillus oryzae* and *Aspergillus niger*. Stable at physiological pH, dHBAD from *Aspergillus* shows high specificity towards 2,3-dHBA and does not decarboxylate its analog 2,3,4-trihydroxybenzoic acid (2,3,5-THBA). Interestingly, dHBAD from *Trichosporon cutaneum* exhibited a broad substrate specificity and decarboxylated 2,3,5-THBA unlike dHBAD from the *Aspergillus* species (Santha et al., 1996).

Halvorson (1963) demonstrated that a species of *Aspergillus* successfully decarboxylated 2,4-dihydroxybenzoic acid to form resorcinol as an intermediary step in

the metabolism of 2,4-dihydroxybenzaldehyde. The 2,4-dHBA was metabolized to form resorcinol after 17 days and was completely metabolized after 29 days.

Category 2 substrates such as 3,4,5-trihydroxybenzoic acid (gallic acid), 3,4-dihydroxybenzoic acid (o-protocatechuic acid), and 4-hydroxybenzoic acid (p-hydroxybenzoic acid) (Figure 10) have a phenolic hydroxyl *para* to the carboxylic acid group but lack the *ortho* hydroxyl group. Decarboxylases for these Category 2 substrates are still of interest, however, due to the variability of enzyme specificity.

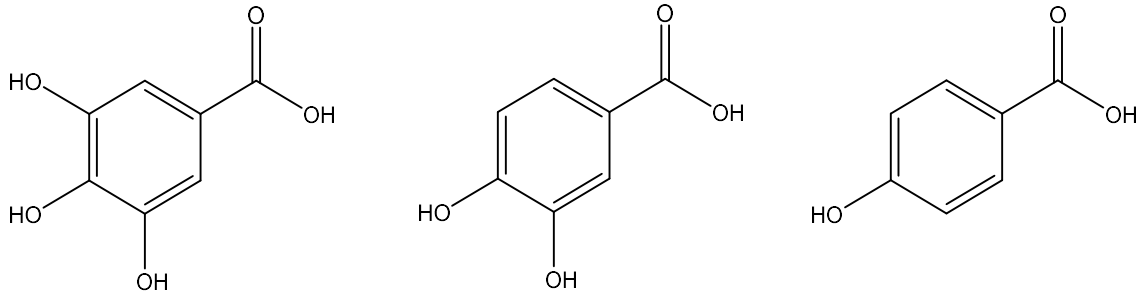


Figure 10: Structures of Category 2 substrates.

An enzyme able to decarboxylate *o*-pyrocatchuic acid (3,4-dihydroxybenzoic acid), a Category 2 substrate, was purified from a member of the *Aspergillus* genus, *Aspergillus niger* (Subba, Moore, & Towers, 1967). *Klebsiella aerogenes,* previously known as *Enterobacter aerogenes,* expresses *p*-hydroxybenzoic acid decarboxylase (PHBD) which decarboxylates gentisic acid (Category 1), *p*-hydroxybenzoic acid (PHB), gallic acid (3,4,5-trihydroxybenzoic acid), and protocatechuic acid. Two days were required to decarboxylate a majority of PHB, gallic acid, and protocatechuic acid and four days for gentisic acid. A permeability barrier was observed for PHB due to the

increase in activity between intact cells and those which had been lysed with sonication. However, the optimum conditions for this enzyme (pH 6) was below human physiological pH (Grant & Patel, 1969).

Nakajima, Otani, & Niimura (1992) isolated *K. pneumoniae* and *Streptococcus faecalis* for the study of decarboxylase enzymes. In their study, they were able to demonstrate that, at an optimal pH of 6, *K. pneumoniae* possessed an enzyme capable of catalyzing the decarboxylation of gallic acid, a Category 2 substrate. Similarly, a decarboxylase enzyme from *S. faecalis* was able to decarboxylate both gallic acid and protocatechuic acid. Optimal conditions for this decarboxylase were found to be a pH of 6.6 and a temperature of 45 °C. Researchers were able to show that for every one molecule of gallic acid, one molecule of 1,2,3-trihyroxybenzene was formed, suggesting a 1 to1 relationship between the substrate gallic acid and its decarboxylated product from the decarboxylase enzyme partially purified from *S. faecalis.*

Three strains of *Lactobacillus brevis* were shown to decarboxylate gallic and protocatechuic acid (Curiel, Rodríguez, Landete, Rivas, & Muñoz, 2010). Cultures were incubated with a variety of 1 mM phenolic acids for 10 days at 30 °C to induce the decarboxylase. Only those incubated in the presence of gallic acid decarboxylated gallic acid and those incubated in the presence of protocatechuic acid decarboxylated protocatechuic acid. Following the observation of decarboxylase activity from *L. brevis,* a phenolic acid decarboxylase (*PAD*) gene was identified, cloned and characterized by Landete et al. (2010) showing an optimum activity at pH 6 and 22 °C.

Compounds which have well-characterized decarboxylases but are lacking much of the structural similarity to CBDA as in Category 1 and 2 are classified as Category 3

substrates such as *p*-coumaric acid, ferulic acid, and caffeic acid (Figure 11), all of which are derivatives of cinnamic acid.

Decarboxylases encoded by *PadC* in *Bacillus subtilis* were shown to display substrate-specificity for decarboxylation of ferulic, *p*-coumaric, and caffeic acid. These three acids are the three substrates of PAD (Duy et al., 2007). Studies from Cavin, Dartois and Divies (1998) showed that induction of the PAD enzyme must occur for decarboxylase activity to be observed in *B. subtilis*. Cell cultures were induced with ferulic, *p*-coumaric, and caffeic acid. All substrates were shown to induce decarboxylase activity, with caffeic acid having the lowest activity.

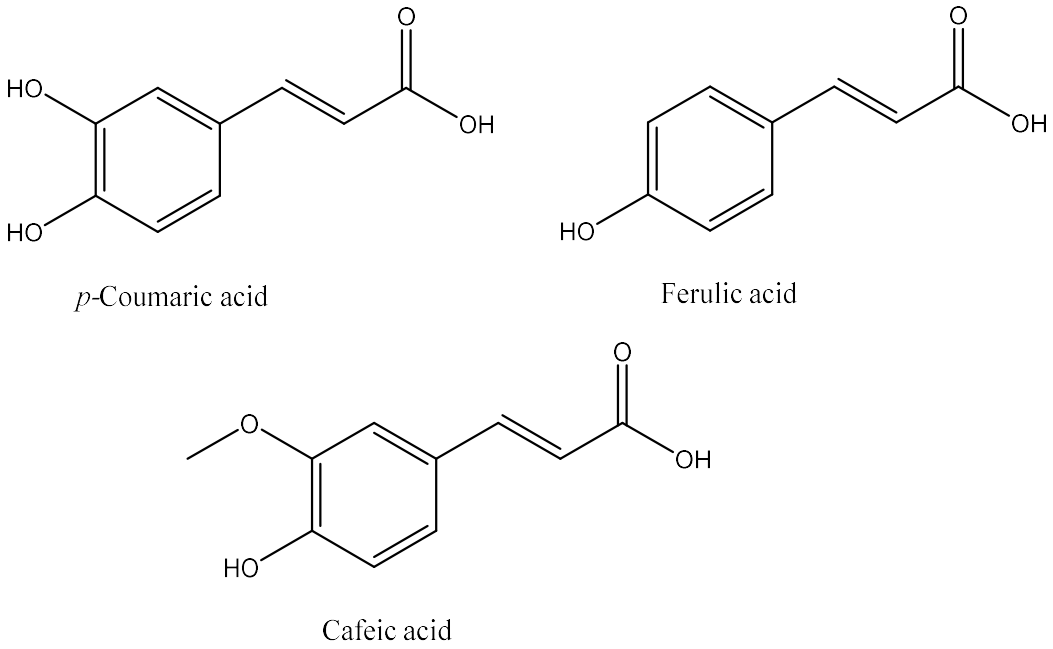


Figure 11: Structures of Category 3 substrates.

Category 4 encompasses miscellaneous substrates which have the least structural similarities to CBDA. A mixed culture of marine bacteria isolated from marine sediment (ON-7), *Bacillus subtillis* (Strain FO) and *Pseudomonas testosterone* were all cultured to examine the metabolism of phthalic acids (Taylor & Ribbons, 1983). The mixed culture

ON-7 was incubated with various phthalic acids for 4 hours at 30 °C. The culture was able to decarboxylate phthalic acid, 4-hydroxyphthalic acid, 3-fluorophthalic acid, 4-chlorophthalic acid, and 4,5-dihydroxyphthalic acid (Figure 12). *B. subtilis* was also incubated with various phthalic acids for 20 hours at 30 °C and was able to decarboxylate all substrates except the 4,5-dihydroxyphthalic acid. The *P. testosterone* culture was also incubated with phthalic acids for 24 hours at 30 °C; however, this cell line only decarboxylated 4-hydroxyphthalic acid and 4,5-dihydroxyphtalic acid.

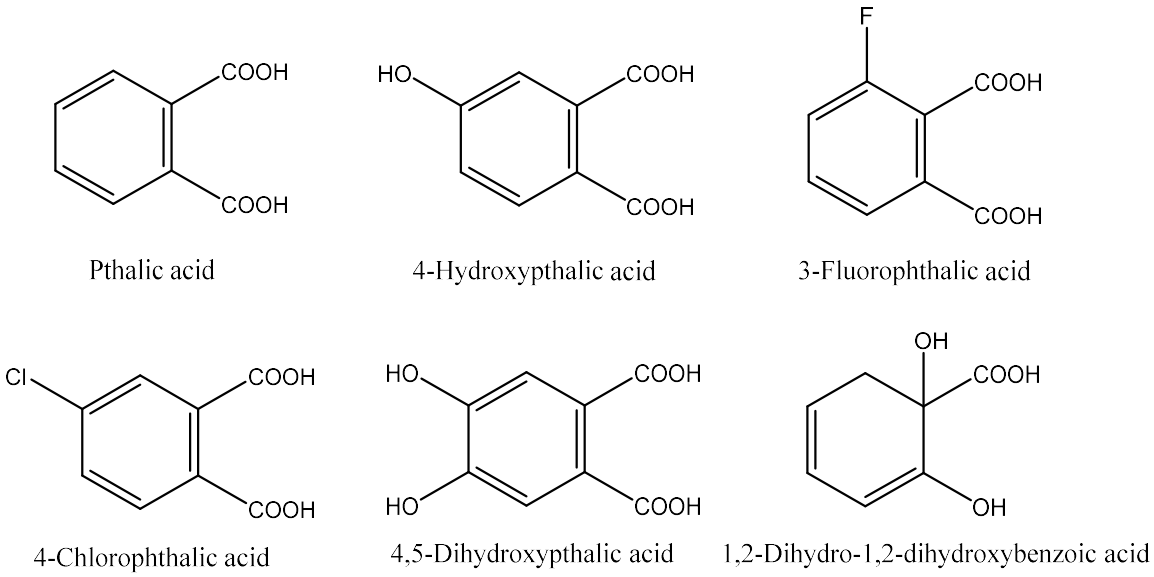


Figure 12: Structures of Category 4 substrates.

An enzyme which has been shown to remove the carboxylic acid *ortho* to a hydroxyl group from 1,2-dihydro-1,2-dihydroxybenzoic acid (Figure 12) is 1,2-dihydro-1,2-dihydroxybenzoic acid dehydrogenase (Reiner, 1971). This enzyme catalyzes the final step in the conversion of 1,2-dihydro-1,2-dihydroxybenzoic acid to catechol and has been characterized from *Pseudomonas putida*, *Pseudomonas cepacian*, *Acinetobacter calcoaceticus,* and *Alcaligenes eutrophus* (Reiner, 1971).

Another enzyme which has been characterized from *P. putida* in addition to that for 1,2-dihydro-1,2-dihydroxybenzoic acid is benzoylformate decarboxylase (BFD) which catalyzes the decarboxylation of benzoylformate to benzaldehyde (Figure 13) (Iding et al., 2000). Although seeming highly specific to the substrate benzoyl formate, the decarboxylase showed minimal activity towards other carboxylic acid groups, in a pH range viable for the human body (pH 7). Important to note is that the purified enzyme did show stability at temperatures up to 60 °C for 2 hours.

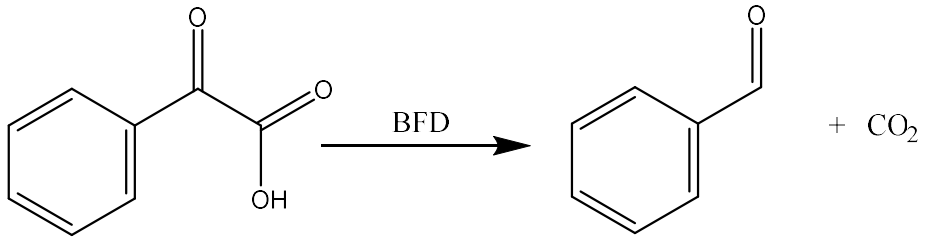


Figure 13: Decarboxylation of benzoylformate to benzaldehyde.

In addition to the aforementioned body of literature, research conducted in our laboratory supports the decarboxylation of cannabinoid model compounds (Cribbs, 2014). Three bacterial species, *K. pneumonia, E. aerogenes,* and *P. putida* displayed decarboxylase activity for various substrates. *P. putida* was incubated with 2-hydroxy-4-methoxybenzoic acid for one hour, after which time the presence of the decarboxylation product, 3-methoxyphenol, was observed with intact cells. In contrast, incubation of *E. aerogenes* in the presence of PHB produced the decarboxylation product, phenol, after 20 minutes with both intact and lysed cells. In this case, induction was not necessary prior to incubation for decarboxylase activity; however, the amount of phenol produced was less than when induced. Finally, *K. pneumonia* was observed to decarboxylate PHB to

phenol following incubation for 30 minutes. After 60 minutes a marked decrease in PHB and increase in phenol was shown (Cribbs, 2014).

Based on the reviewed literature and previous work by our research group, seven cell lines were chosen for screening: *Aspergillus niger, Aspergillus clavatus, Bacillus subtilis, Enterobacter (Klebsiella) aerogenes, Klebsiella pneumonia, Penicillium chrysogenum*, and *Pseudomonas putida. K. pneumonia, E. aerogenes,* and *P. putida* displayed decarboxylase activity for various model compounds in previous studies by our research group and thus were carried forward to be screened with actual cannabinoids. *Bacillus subtilis* has a well-characterized PAD enzyme which decarboxylates a variety of compounds from Categories 1-4 and so was also chosen to be screened with cannabinoids. The same rationale was used in choosing those cell lines from the *Aspergillus* and *Penicillum* genera. The cells chosen contained decarboxylases which covered a variety of structural variations from that of CBDA to have a more complete understanding of which enzyme binding sites might be most compatible with the structure of CBDA.

Cell-in-a-Box®

The Cell-in-a-Box® live cell encapsulation technology was developed by Austranova, an Austrian biotechnology company, and employed by PharmaCyte Biotech as a delivery system of cells which will potentially convert prodrugs to active drugs for the treatment of a variety of different diseases. This delivery system is currently in clinical trials for treatment of pancreatic and breast cancer, as well as diabetes. PharmaCyte Biotech's model for the treatment of cancer involves encapsulating genetically-engineered human cells that convert an inactive chemotherapy drug into its

active or “cancer-killing” form (PharmaCyte, 2017). Cells are encapsulated though a reaction of two polymer coating steps in which the cells are mixed with one polymer and then the mixture is introduced to the next polymer using a droplet-forming machine, where the capsule membrane complex forms immediately around the droplets containing cells, resulting in capsules with a diameter of 0.7-0.8 mm which can encapsulate as many as 10,000 live cells (PharmaCyte, 2017).

The small, spherical capsules, made of a bio-inert cellulose-based polymer, are designed to retain the engineered cells due to size, while preventing immunoglobulins from entering the capsule and destroying the cells. The porous nature of the capsule allows blood flow, which nourishes the living cells inside, and does not block circulation when inserted into the blood stream. In cancer therapies, capsules are implanted as close as possible to the patient’s cancerous tumor which can be done so safely by using needles or catheters. Capsules that have been placed in the body for over two years have not degraded nor caused damage or inflammation to the surrounding tissues (PharmaCyte, 2017).

Cell-in-a-Box® technology is currently being implemented in clinical trials for the treatment of pancreatic cancer with the prodrug ifosfamide, a highly effective chemotherapy drug. However, ifosfamide is typically given intravenously and activated in the liver by cytochrome P450; consequently, the effective dose needed for the desired anti-cancer effects of ifosfamide can cause serious side effects. With the use of Cell-in-a-Box® technology, ifosfamide is given intravenously and converted to its active form when it comes into contact with the encapsulated cells implanted in the blood supply near the tumor instead of being converted in the liver and distributed throughout the body until

reaching the tumor site. A pictorial schematic of this mechanism of action is presented in Figure 14. In clinical trials, patients were able to receive a dose two-thirds less than the usual, which decreased or completely eliminated the undesirable side effects.

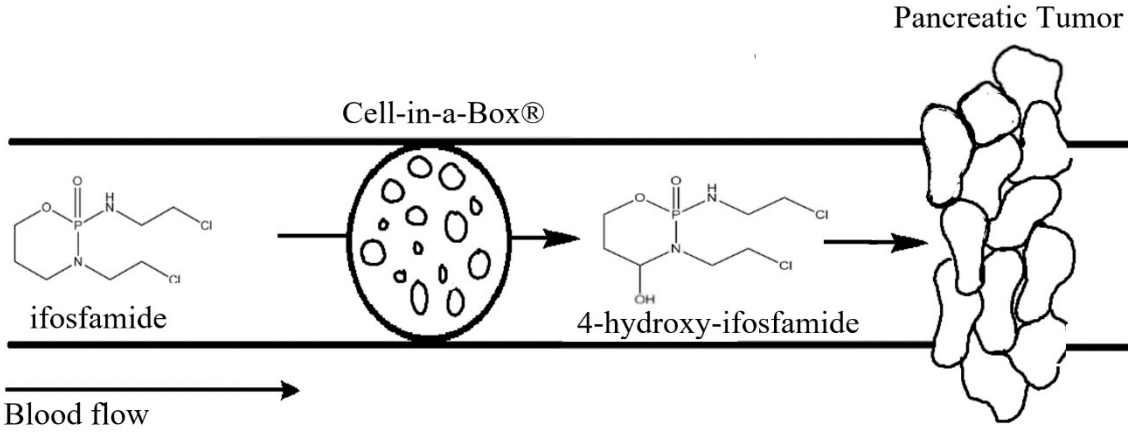


Figure 14: General schematic of Cell-in-a-Box®/Ifosfamid technology.

In a phase 1 and 2 clinical trial, 14 patients were treated with the Cell-in-a-Box®/Ifosfamide therapy. Cell-in-a-Box® capsules, with engineered cells which expressed the P450 system like that of the liver where ifosfamid is usually metabolized, were implanted on day zero. One dose of ifosfamide was given intravenously to patients during the first week (days 2-5) and a second dose was given during the fourth week (days 25-27), both at a dose of 1 g/m^2. Individual results varied from 25-50% reduction in tumor size; however, no patients showed tumor growth and two patients' inoperable tumors (due to size and location in the pancreas) became operable. These results were compared with phase 3 historical data for gemcitabine, a pancreatic cancer treatment currently approved by the FDA. The percentage of one-year survivors using the Cell-in-a-Box® therapy was double that previously reported for patients using gemcitabine, the median survival of patients was substantially increased as compared to gemcitabine, and

in contrast to gemcitabine, no treatment-related side effects were seen with Cell-in-a-Box®/Ifosfamid treatment (Pharmacyte, 2017).

In a phase 2 clinical trial using the Cell-in-a-Box®/Ifosfamid treatment, 13 pancreatic cancer patients were administered Cell-in-a-Box® technology in the same manner as in the phase 1/2 trials, however a dose of 2 g/m^2 was given. The conclusion was that a dose of 1 g/m^2 was more appropriate, since that dose was able to effectively treat the pancreatic tumors while still minimizing negative effects of the typical mode of delivery for ifosfamid. A future trial, awaiting FDA approval, is designed to show that Cell-in-a-Box® plus low-dose ifosfamide therapy can serve as an effective and safe consolidation chemotherapy for patients whose tumors no longer respond after four to six months of therapy with Abraxane® plus gemcitabine (PharmaCyte, 2017).

In addition to trials with human pancreatic cancer, a veterinary phase 1/2 trial using dogs with breast cancer has been conducted. Cell-in-a-Box® capsules containing cells with high CYP2B1 activity were used in combination with cyclophosphamide. Because it is often used to treat breast cancer in humans as well as mammary tumors in dogs, cyclophosphamide was chosen rather than ifosfamide. As in the pancreatic cancer clinical trials in humans, the capsules showed no side effects or irritation at the implanted sites (PharmaCyte, 2017).

A total of 16 dogs were enrolled in the preclinical trial. Ten dogs were treated with the Cell-in-a-Box®/cyclophosphamide combination, one of which had two tumors. Six of the 11 tumors from the dogs treated with the encapsulated cells/cyclophosphamide combination showed a partial remission (PR, regression of initial tumor mass by less than 50%) while the other five tumors showed stable disease (SD, regression of the initial

tumor mass by less than 50% or its enlargement by less than 25%). One group of six dogs, one of which had two tumors, was treated intravenously with cyclophosphamide alone at a dose of 7 mg/kg of body weight. Of the seven tumors in the six dogs that received cyclophosphamide alone, six tumors showed SD and the other exhibited progression of disease (PD, increase in initial tumor mass of at least 25% or the occurrence of new tumors). The median tumor size reduction of the tumors in the ten dogs treated with the combination Cell-in-a-Box®/cyclophosphamide was 53%. This is in contrast with only a 21% reduction of the size of the tumors in the dogs treated with cyclophosphamide alone (Michalowska et al., 2014). The results of this study suggest that the combination of the Cell-in-a-Box®/cyclophosphamide treatment may prove to be beneficial not only for the treatment of mammary cancers in the veterinary setting but may also be translated into an effective treatment of breast cancer in humans. This supports the application of the Cell-in-a-Box® technology as a treatment platform.

Summary

With the support from the studies reviewed in this chapter, this research aimed to isolate the phytocannabinoid cannabidiolic acid from *Cannabis sativa* plant material and to screen microbial cell lines which could potentially decarboxylate CBDA to CBD. With the broad review of many different *in vitro* and *in vivo* studies citing the effects of CBD on various cancer cell lines, this natural product clearly could be the basis of a targeted cancer therapy. Cannabidiol is already an FDA-approved drug in other medicinal contexts and its high potential for other therapies could be harnessed if an appropriate manner of administration was developed. One approach involving Cell-in-a-Box® begins with identifying a microbial cell line capable of enzymatic conversion of

the inactive prodrug cannabidiolic acid to cannabidiol by performing cell assays on a variety of species from the literature discussed. Ultimately, Cell-in-a-Box® technology could provide a potential mechanism of delivery for a genetically-engineered cell containing the gene necessary for an enzyme capable of catalyzing the conversion of prodrug to active drug.

CHAPTER III

METHODOLOGY

Chemicals and Reagents

Cannabidiolic acid (CBDA) (1244-58-2), cannabidiol (CBD) (13956-29-1), and phytocannabinoid mixture 1 (18791) were obtained from Cayman Chemical Company (Ann Arbor, Michigan). Chloroform-d (865-49-6) and formic acid (64-18-6) were obtained from Acros Organics (Geel, Belgium). HPLC-grade acetonitrile (75-05-8), absolute ethanol (64-17-5), n-pentane (109-66-0) and potassium hydroxide (1310-58-3) were obtained from Fisher Scientific (Fair Lawn, New Jersey). Ammonium formate (540-69-2), HPLC-grade methanol (67-56-1), Δ^9-THC (1972-08-3) and salicylic acid (69-72-7) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). The sources for the remaining chemicals and reagents are as follows: Tryptic soy broth (470015-844) (BD Sparks, MD); sabouraud-dextrose broth (1466366) (EMD, Darmstadt, Germany). *Cannabis* plant material (RTI log number 13784-1114-18-6, reference number SAF 027355, 6.5% CBD(A), 3.5% THC(A)) was obtained from the National Institute on Drug Abuse. HPLC-grade water (18.2 MΩ) was purified in-house by passing reverse osmosis purified water through a Milli-Q® Advantage A 10 Ultrapure Water Purification System.

Cells

Seven cell lines were screened for decarboxylase activity with CBDA. *Aspergillus clavatis* (1007) and *Apergillus niger* (16888) were purchased from the

American Type Culture Collection (Manassas, VA). *Bacillus subtilis* (470176-524), *Enterobacter (Klebsiella) aerogenes* (470179-294), *Klebsiella pneumoniae* (470179-122), *Penicillium chrysogenum* (470176-388) and *Pseudomonas putida* (470179-092) were obtained from VWR International (Randor, PA) via Ward's Science Co. (Rochester, NY).

Preparation of Solutions

All mass measurements were made using an Ohaus Explorer Pro analytical balance. The pH of buffer solutions was measured using a Fisher Scientific Accumet® AB150 pH meter. The meter was calibrated using buffers obtained from Fisher Scientific at pH 4.0 (SB-101-500), 7.0 (SB-107-500), and 10.0 (SB-115-500). All solvents were filtered prior to use with the HPLC. Aqueous solvents and methanol were filtered through a nylon filter (0.22 μm) and all other organic solvents were filtered through a Teflon filter (0.22 μm).

Ammonium formate (20.0 mM, pH 3.20) preparation. To prepare 1.00 L of 20.0 mM ammonium formate, 1.2612 g of ammonium formate were added to a 1000-mL volumetric flask and diluting to volume with HPLC-grade water. Formic acid (20.0 mM) was prepared by adding 0.3835 mL of 96% formic acid to a 500-mL volumetric flask and diluted to volume with HPLC-grade water. The 20.0 mM formic acid solution was added to the 20 mM ammonium formate solution until a pH of 3.20 was observed on the pH meter.

Cannabidiolic acid (2.0 mg/mL) stock solution preparation. To prepare 700 μL, 1.4 mg of CBDA were added to a vial and dissolved in 700 μL of absolute ethanol, added by a micropipette.

Cannabidiol (2.0 mg/mL) stock solution preparation. To prepare 1.05 mL, 2.1 mg of CBD were added to a vial and dissolved in 1.05 mL absolute ethanol, added by micropipette.

Standard curve solutions for cannabidiolic acid. Five 200 µL standards with concentrations of 0.10, 0.50, 1.0, 1.5, and 2.0 mg/mL were prepared by making appropriate dilutions of the stock solution using a variety of Rainin Pipetman micropipettes. The standards were diluted to a volume of 200 µL with absolute ethanol. All standards were prepared in triplicate.

Standard curve solutions for cannabidiol. Five 200 µL standards with concentrations of 0.10, 0.50, 1.0, 1.5, and 2.0 mg/mL were prepared by making appropriate dilutions of the stock solution using a Rainin Pipetman micropipette. The standards were diluted to a volume of 200 µL with absolute ethanol. All standards were prepared in triplicate.

Induction solution preparation. The appropriate mass of the induction substrate, according to assay, was dissolved in an appropriate solvent in a volumetric flask depending on the substrate and the desired volume. For example, salicylic acid was prepared as the potassium salt by dissolving the appropriate amount in an equimolar + 10% equivalent of KOH in a 250-mL volumetric flask. An additional 25 mL of absolute ethanol were added then diluted to volume with HPLC-grade water.

Construction of Calibration Curves

Calibration curves for both CBDA and CBD were constructed for use to determine the concentration of both CBDA present and CBD formed, if any, from the signal produced during HPLC analysis. A 2.0 mg/mL stock solution of each compound

was prepared and then diluted with absolute ethanol to make solutions of 1.5, 1.0, 0.50, and 0.10 mg/mL. Each standard was prepared in triplicate and analyzed using the analytical HPLC method. The area under the curve of the analyte peak was averaged across the triplicate solutions and plotted in Microsoft Excel against concentrations to generate a standard curve.

Isolation of Cannabidiolic Acid from Cannabis Plant Material

Initially, 5.00 g of *Cannabis* plant material, containing a reported 6.5% CBD(A) content, obtained from the National Institute for Drug Abuse was freeze-dried and pulverized with a mortar and pestle prior to extraction. The dry material was placed in a filter paper thimble which was then placed into the main chamber of a Soxhlet extractor. The Soxhlet extractor was placed onto a round-bottom flask containing 100 mL of pentane which was then heated to a gentle reflux in a continuous solid-liquid extraction for three hours. After extraction, the pentane was removed by rotary evaporation to recover a dark green-brown, oily and viscous extract containing CBDA and other cannabinoids. The resulting oil was resuspended in 15 mL ethanol and gravity filtered to remove any insoluble solids. CBDA was isolated from the resulting solution as described in the Preparative Method for Purifying CBDA from a Crude *Cannabis* Extract outlined in the Instrumentation section of Methodology.

Cell Culture

Cells were cultured in 500-700 mL of appropriate growth medium and incubated at room temperature or 37 °C according to species. Cells were induced for 24-72 hours with model compounds known for inducing decarboxylase activity. Treatments for each assay are listed in Table 9 (Appendix A). For example, salicylic acid was added to a

culture of *Pseudomonas putida* to give a final concentration of 1 mM and incubated for 72 hours to induce expression of decarboxylase enzymes. Following induction, cells were centrifuged in 50-mL conical tubes at 4000*g* for 10 minutes to collect all cells in a pellet. Wet cell mass of the final cell pellet was obtained in mg/mL. Cells were resuspended in 30 mL of fresh culture medium and divided between two 50-mL conical tubes. One sample was sonicated to lyse the cells or used intact for assessing decarboxylase activity. Sonication was performed using a Branson Digital sonifier using a program designed to sonicate the sample at 40% amplification using one second bursts followed by a one second pause for a total of twenty seconds. Cells were on ice during sonication to ensure that the sample remained cool. The other sample, used as control, was autoclaved. All samples were kept on ice until screened for enzymatic activity.

Cell Assay for Decarboxylase Activity

Each strain of microbial cells was grown according to the procedure outlined in the Cell Culture section of Methodology. Cells were assessed for enzymatic decarboxylation of CBDA alongside an autoclaved control. Cells were assayed at a range of concentrations of CBDA from 0.23-0.36 mM by making solutions of 1.1-1.75 mg of CBDA in 100 µL of absolute ethanol and adding to the incubation medium. Specific concentrations of CBDA are given in Table 9 (Appendix A). Addition of the CBDA solution (100 µL) to the incubation flask was followed by the addition of 15.0 mL of cell suspension. The solutions were incubated in a constant-temperature, shaking water bath from VWR International at 36 °C and shaken at 60 rpm for the duration of the assay. Incubation media was removed in 2.0 mL aliquots at time = 0, 1, 2, 3, 4, 5, and 6 hours and immediately extracted with 5.0 mL of pentane containing 0.5 mL absolute ethanol by

vortexing with the 2.0 mL aliquot of incubated media. The cannabinoid-containing pentane layer was separated from the cell suspension using centrifugation; the pentane layer was transferred into a separate vial and evaporated to dryness using nitrogen gas. The residue was resuspended in 100 µL of absolute ethanol. A 20 µL aliquot of the ethanol solution was loaded into the HPLC 10 µL loop injector and analyzed for the presence of CBD using HPLC in accordance to the Analytical Method for Monitoring Conversion of CBDA to CBD outlined in the Instrumentation section of Methodology.

Instrumentation

Analytical High Performance Liquid Chromatographic Method for Monitoring Conversion of Cannabidiolic Acid to Cannabidiol

HPLC analyses were performed using a Shimadzu (Tokyo, Japan) VP equipped with a SPD-M10A diode array detector, a Shimadzu SCL-10A system controller, with a DGU-14A degasser, one LC-10AT pump, and a FCV-10AL Low Pressure Valve Module. Injection was through a 10 µL injection loop. Separations were obtained on a reverse phase analytical column (Luna Omega 5 µm Polar C18 100 LC column 150 × 4.6 mm with a 4.0 × 3.0 mm guard column) obtained from Phenomenex which was housed in a Shimadzu CTO-10AS column oven set to 30 °C.

A 20 minute long, two-solvent gradient method was used with 20 mM ammonium formate buffer, pH 3.20 (solvent A) and HPLC-grade acetonitrile (solvent B). The gradient steps began with 60% solvent B increased to 95% solvent B over a nine-minute period, held at 95% for three minutes and then reduced back to 60% solvent B by minute sixteen and held at 60% until the end of the run. Flow rate was maintained at

1.2 mL min^{-1}. The diode array detector was set to scan in 1 nm increments from 190 – 800 nm.

CBDA and CBD were identified by comparing their retention times to those of reference standards. Under these conditions, the retention times of CBDA and CBD were 7.0 and 8.1 minutes, respectively. Chromatograms were generated with CLASS-VP 7.2.1 SP1 software.

Preparative High Performance Liquid Chromatographic Method for Purifying Cannabidiolic Acid from a Crude *Cannabis* Extract

A Shimadzu VP HPLC was used to develop a preparative method for purifying CBDA from a crude *Cannabis* extract. The HPLC was equipped with a CBM-10AW communications bus module and a SPD-10A UV-VIS detector DGU-14A degasser, two LC-10AT pumps, and a 1.00 mL injection loop. Separations were obtained on a preparative reverse phase column (Luna Omega 5 μm Polar C18 100 LC column 150×21.2 mm with a Polar C18 SecurityGuard prep cartridge, 15×21.2 mm) obtained from Phenomenex, housed in a Shimadzu CTO-10A column oven set to 30 °C. A Gilson FC-100 microfractionator was connected to the HPLC effluent outlet and used to collect the separated compounds.

A 90 minute long, two-solvent gradient method was used with 20 mM ammonium formate buffer at 3.20 pH (solvent A) and HPLC-grade acetonitrile (solvent B). The gradient steps began with 50% solvent B held for five minutes and increased to 95% solvent B over a 55-minute period, 95% solvent B was maintained for 10 minutes, and then decreased back to 50% over the remaining 20-minute period. Flow rate was

maintained at 4.5 mL min^{-1}. The two channels of the UV-VIS detector were set to 220 nm and 274 nm. Under these conditions, the retention time of CBDA on the preparatory column was found to be 46 minutes. Chromatograms were generated with CLASS-VP 7.2.1 SP1 software.

CBDA was identified by collecting major peaks using the Gilson FC-100 microfractionator set to collect in one-minute increments beginning 40 minutes into the run. The test tubes containing the major peaks were collected from the fractionator. Test tubes which contained fractions of the same peak were combined. Each sample representing a peak in the chromatogram was placed into a 50-mL round-bottom flask. Cannabinoids were recovered from the HPLC solvent solution by first rotary evaporating the acetonitrile and then back extracting the cannabinoids from the remaining ammonium formate solution into 5.0 mL pentane. The pentane layer was transferred with a Pasteur pipette to a test tube. The pentane was evaporated to dryness using nitrogen gas and the resulting residue was resuspended in 1.0 mL ethanol.

Infrared Spectroscopy Procedure

A Thermo Scientific Nicolet iS5 IR equipped with an iD5 ATR accessory and zinc selenide crystal was used to acquire IR spectra using the OMNIC software. A 1.0 mg sample of CBDA recovered from the preparative HPLC was applied to the surface of the crystal. After the pressure arm was lowered and secured, the sample was analyzed. Obtained spectra were compared to standard spectra from a study by Hazekamp, Peltenburg, Verpoorte & Giroud (2005).

^{1}H- and ^{13}C Nuclear Magnetic Resonance Procedure

A Bruker 400 MHz NMR was used with TOPSPIN 1.3 software program to generate all NMR spectra. Confirmatory ^{1}H- and ^{13}C-NMR spectra were acquired for cannabidiolic acid isolated using the outlined preparative HPLC method. Since the recovered CBDA was initially dissolved in 1.0 mL ethanol, the sample was evaporated to dryness with nitrogen gas and the residue (3.7 mg) was resuspended in 600 µL of deuterated chloroform for NMR analysis. Obtained spectra were compared to standard spectra of cannabinoids from a study by Choi et al. (2004).

CHAPTER IV

RESULTS AND DISCUSSION

This study had a dual purpose, to isolate CBDA from the plant *Cannabis sativa* and to identify a microbial cell line which possesses a decarboxylase enzyme capable of converting CBDA to CBD. Before various cells could be screened for decarboxylase activity, the optimal conditions for the chromatographic column and mobile phase had to be confirmed to ensure optimal separation and identification of the cannabinoids. This analytical method was employed to monitor any enzymatic conversion of CBDA to CBD in the enzymatic assays and as a confirmatory method for preparatory isolation of CBDA. Once developed, cell lines could be monitored for CBDA decarboxylase activity. The following sections discuss results from HPLC methodology development, CBDA isolation, and enzymatic assays.

Analytical Method for Monitoring Conversion of Cannabidiolic Acid to Cannabidiol

Model compounds from previous studies were separated by HPLC on a Tosohaus C_{18} column using an isocratic method consisting of 35% methanol and 65% 0.5 M potassium acetate buffer, pH 3.0, with a flow rate of 1.0 mL/min at room-temperature (Cribbs, 2014); however, this method was not suitable since it was not able to baseline resolve CBDA and CBD well. Separating these two coumpounds was particularly challenging because there is such a small difference in the chemical formula, molecular

weight, and the physical and chemical properties of CBDA and CBD. The reverse phase polar C18 (Luna Omega 5 µm Polar C18 100 Å, LC Column 150 x 4.6 mm) column was obtained from Phenomenex who suggested an isocratic mobile phase of 25% water + 0.1% formic acid and 75% acetonitrile + 0.1% formic acid reported in their applications. They also reported a fifteen minute-long gradient method using 20 mM ammonium formate, pH 3.2 (solvent A) and acetonitrile (solvent B) for a similar column with different dimensions (Luna Omega 1.6 µm C18 100 Å, LC Column 100 x 2.1 mm). This method began by increasing solvent B from 60% to 95% over 12 minutes, holding for one minute, and then decreasing solvent B back to 60% during the remaining time at a flow rate of 0.4 mL min^{-1} at 40 °C. The gradient approach was taken; however results reported by Phenomenex® could not be replicated with the difference in columns. The initial concentration of solvent B was increased from 5% to 60%, flow rate was increased to 1.2 mL min^{-1}, and column temperature was decreased to 30 °C. This achieved greater separation of various cannabinoids. The optimized analytical method is described under the Instrumentation section in Chapter III (Methods).

With the optimized analytical HPLC method, standards of CBDA and CBD were analyzed to confirm the identity of analyte peaks from enzymatic assays and CBDA isolation by matching their retention times with that of these standards. The retention times of CBDA and CBD were determined to be 7.0 min (Figure 15) and 8.3 min (Figure 16), respectively. Baseline separation of both compounds was achieved (Figure 17) which allows for the conversion of CBDA to CBD to be monitored. Interesting to note is the difference in retention times of CBDA and CBD when in a mixture than when pure, possibly due to intermolecular interactions such as hydrogen bonding. Cannabidiolic

acid had a retention time of 7.0 min. when pure in an ethanolic solution compared to 7.5 min in an ethanolic solution with CBD. Cannabidiol's retention time in a mixture with CBDA was shifted to 8.3 min from 8.1 min when analyzed with this method.

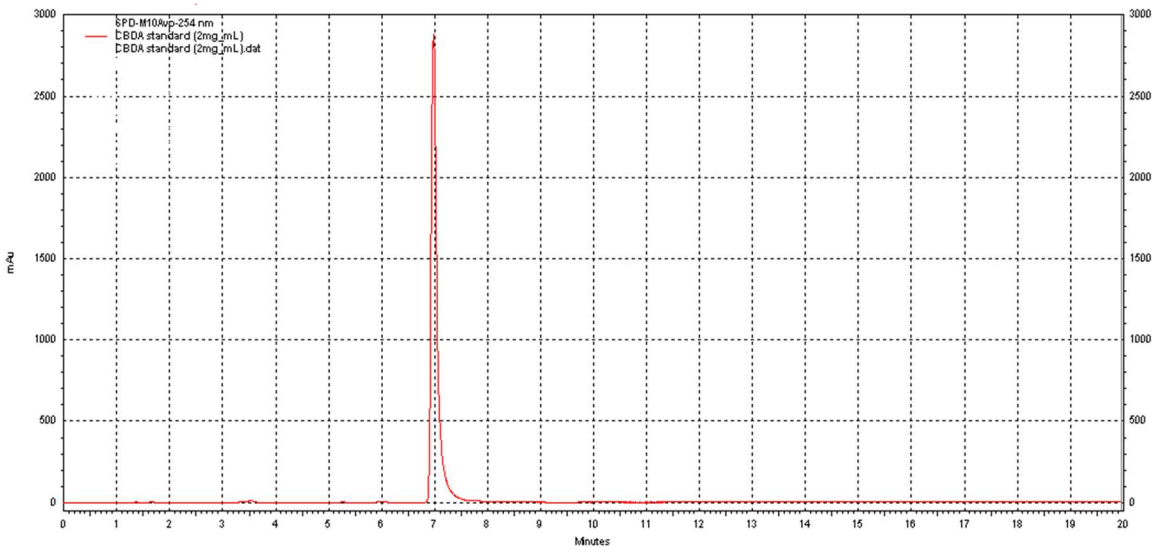


Figure 15: Chromatogram of 2.0 mg/mL CBDA standard using the analytical HPLC method. Retention time 7.0 minutes.

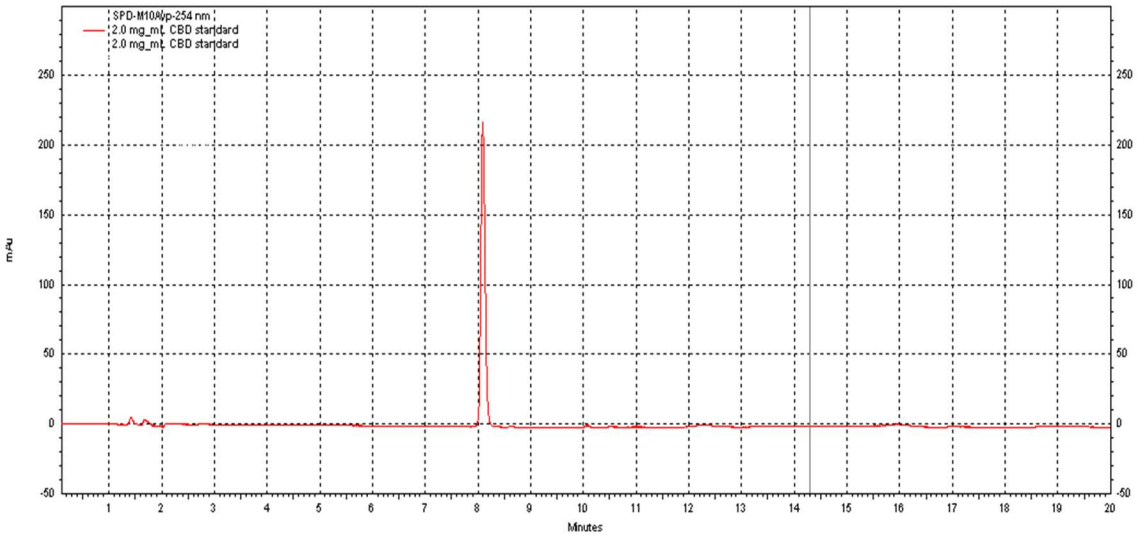


Figure 16: Chromatogram of 2.0 mg/mL CBD standard using the analytical HPLC method. Retention time 8.1 minutes.

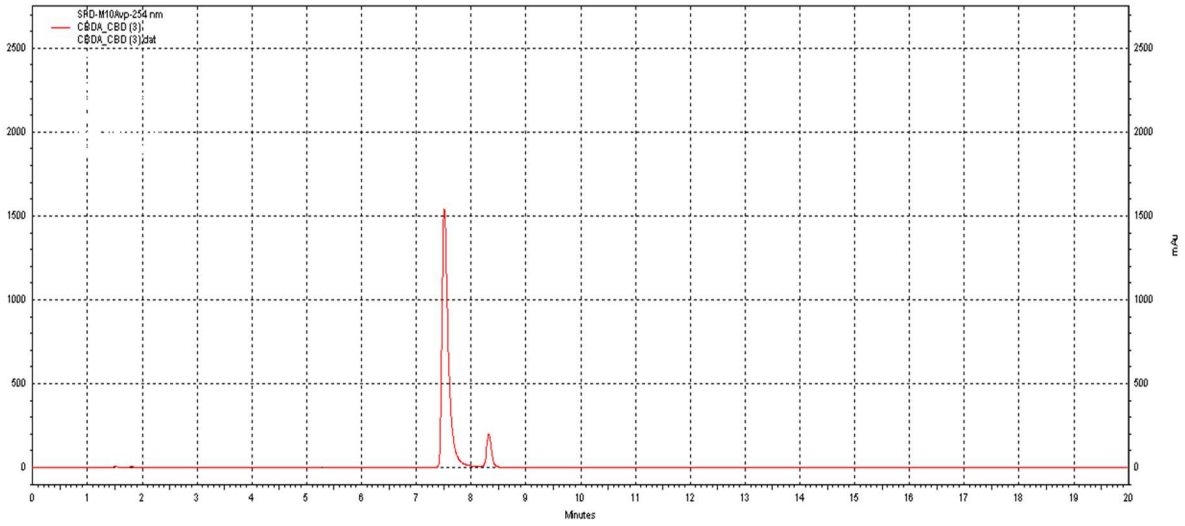


Figure 17: Chromatogram of a mixture of 1.0 mg/mL CBDA and 1.0 mg/CBD using the analytical HPLC method. CBDA retention time 7.5 min, CBD retention time 8.3 min.

Standard curves for both CBDA (Figure 18) and CBD (Figure 19) were constructed to quantify any enzymatic conversion during cell assays based qualitatively on the appearance of the CBD peak. Solutions of CBDA and CBD were prepared in triplicate over a range of concentrations (0.10 – 2.0 mg/mL) and analyzed using the analytical HPLC method. The area under the curve for each run of the same concentration was averaged and plotted against the concentration. Areas and averages for peaks from various concentrations of CBDA and CBD standards are given in Table 1 and Table 2, respectively.

Table 1: Area of Cannabidiolic Acid Peak at Different Concentrations Using the Analytical HPLC Method.

CBDA Concentration (mg/mL)	Trail 1	Trial 2	Trial 3	Average	Standard Deviation
2.0	4.07×10^7	3.90×10^7	4.00×10^7	3.99×10^7	6.96×10^5
1.5	2.92×10^7	3.46×10^7	3.11×10^7	3.16×10^7	2.27×10^6
1.0	2.48×10^7	2.26×10^7	2.08×10^7	2.27×10^7	1.64×10^6
0.5	9.26×10^6	8.30×10^6	8.22×10^6	8.59×10^6	4.72×10^5
0.1	4.72×10^6	1.06×10^6	5.36×10^6	3.71×10^6	1.89×10^6

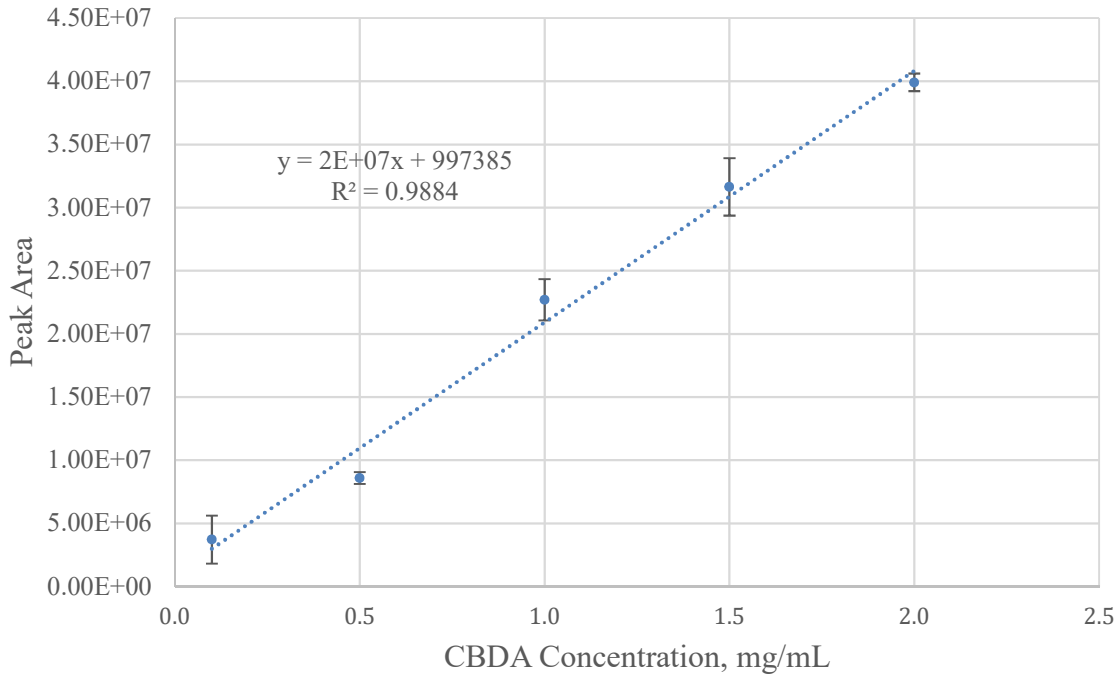


Figure 18 : Standard calibration curve for cannabidiolic acid.

Table 2: Area of Cannabidiol Peak at Different Concentrations Using the Analytical HPLC Method.

CBD Concentration (mg/mL)	Trial 1	Trial 2	Trial 3	Average	Standard Deviation
2.0	2.40×10^7	2.48×10^7	2.38×10^7	2.42×10^7	4.56×10^5
1.5	1.87×10^7	1.87×10^7	1.93×10^7	1.89×10^7	2.42×10^5
1.0	1.70×10^7	1.67×10^7	1.52×10^7	1.63×10^7	7.79×10^5
0.5	7.94×10^6	8.07×10^6	8.02×10^6	8.01×10^6	5.29×10^4
0.1	2.05×10^6	2.15×10^6	1.94×10^6	2.05×10^6	8.57×10^4

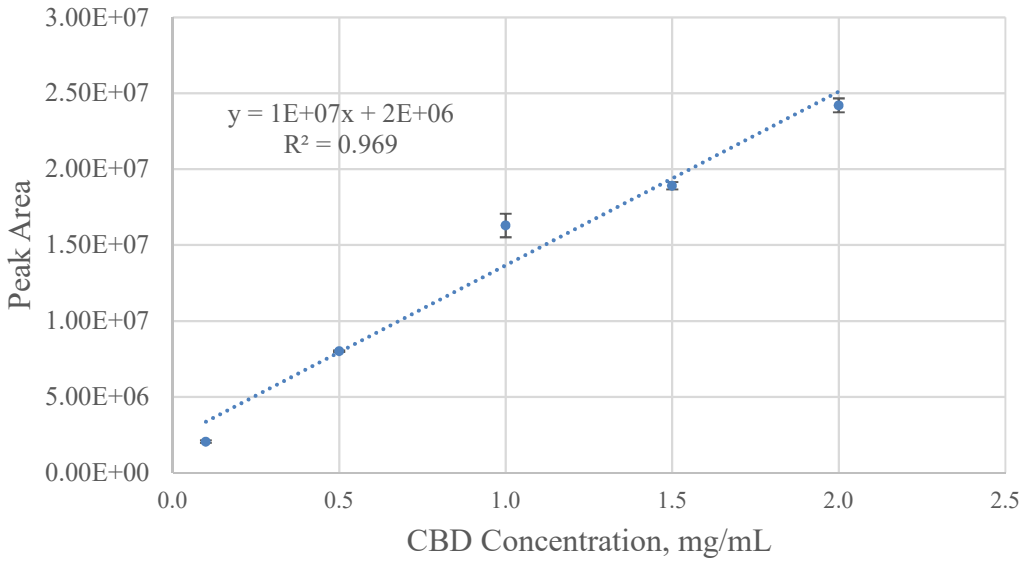


Figure 19: Standard calibration curve for cannabidiol.

Isolation and Characterization of Cannabidiolic Acid

Preparative High Performance Liquid Chromatographic Method for Purifying Cannabidiolic Acid from a Crude Cannabis Extract

In addition to the analytical HPLC method, a preparatory HPLC method for cannabinoids was developed for the purpose of isolating pure CBDA from the crude Soxhlet *Cannabis* extract. Crude extract was prepared from 5.00 g of *Cannabis* containing a reported 6.5% CBDA (0.325 g) as outlined in Methodology. The extract was initially analyzed on the preparatory column using a two-solvent gradient, referred to as Gradient 1 (Figure 20), at 30 °C, with a flow rate maintained at 5 mL min^{-1}, however poor separation was achieved (Figure 21). The length of the gradient step from 25% to 95% acetonitrile was increased from 65 minutes to 95 minutes, while flow rate was decreased to 4.5 mL min^{-1} to make Gradient 2 (Figure 22). This method was able to achieve separation of at least 4 major components (Figure 23).

Because the first compounds from the extract eluted one hour into the run, analysis time was shortened by 60 minutes by increasing the starting percentage of the eluent from 25% to 50% acetonitrile. The temperature and flow rate from Gradient 2 was maintained. The resulting method (Gradient 3, Figure 24) was able to resolve cannabinoids (Figure 25) in a similar manner to Gradient 2 in 30 minutes less and with 32.5% less solvent than the originally suggested method using Gradient 1. Both time and materials can be costly so optimizing the method in this way was ideal to save valuable

resources with very simple adjustments. The optimized preparatory method is described under “Instrumentation” in Chapter III (Methods).

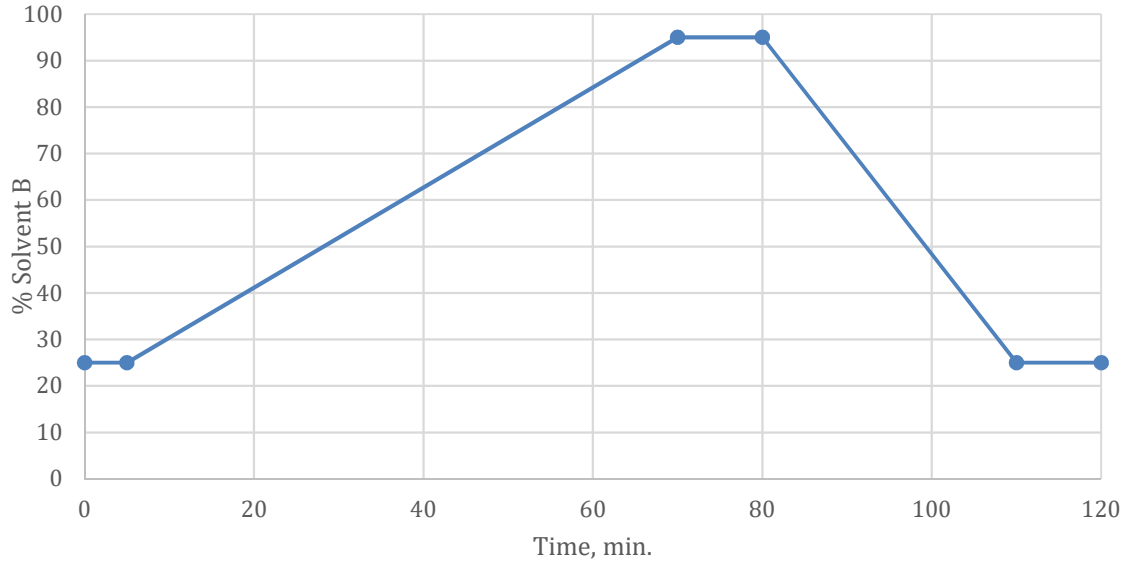


Figure 20: Gradient Method 1

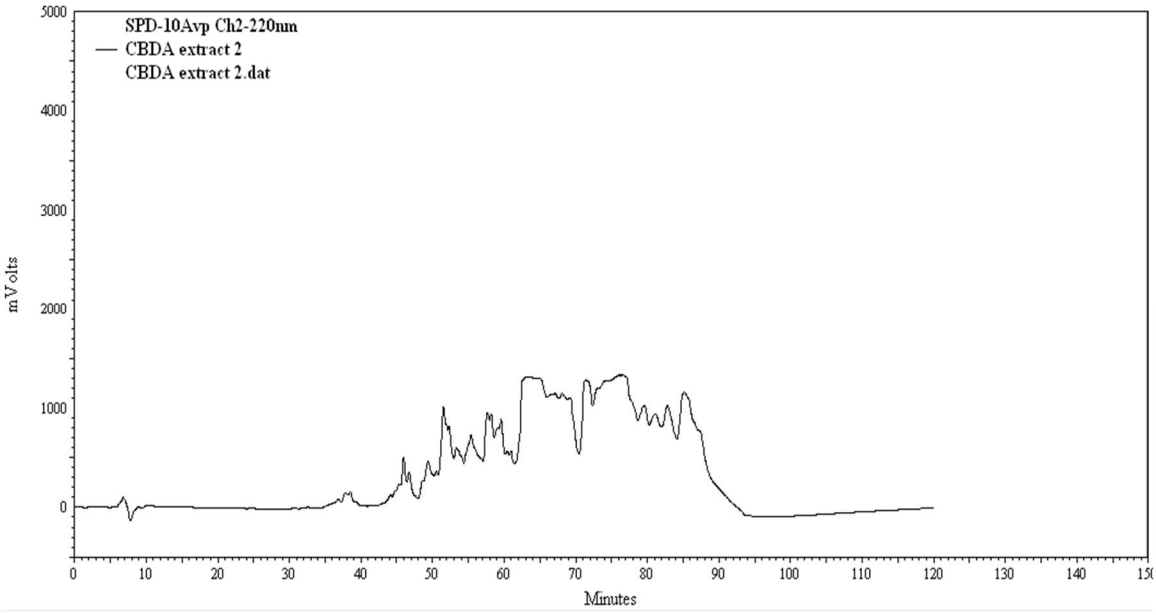


Figure 21: Chromatogram of CBDA extract from Soxhlet extraction using the preparatory HPLC method with Gradient 1.

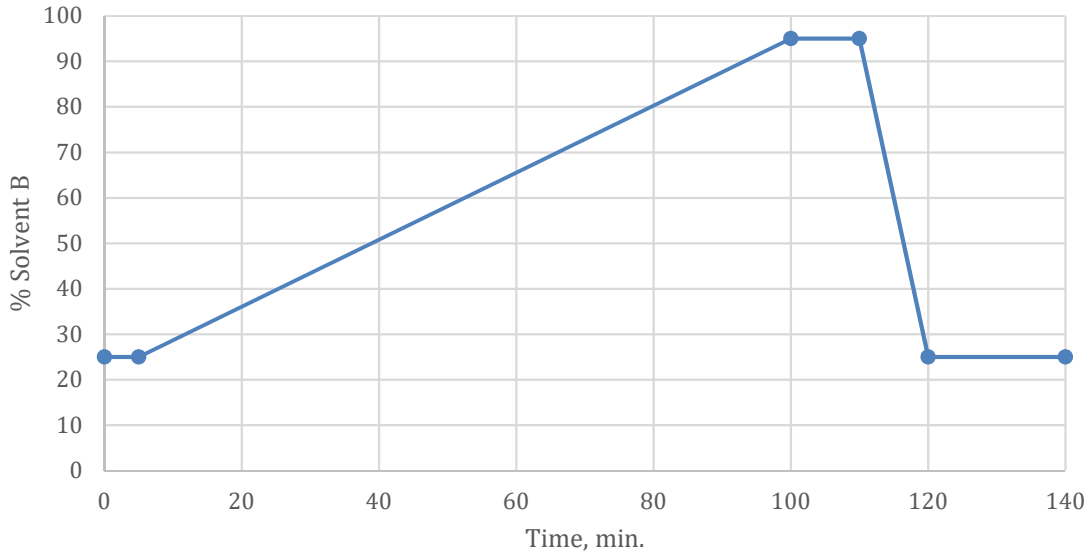


Figure 22: Gradient Method 2

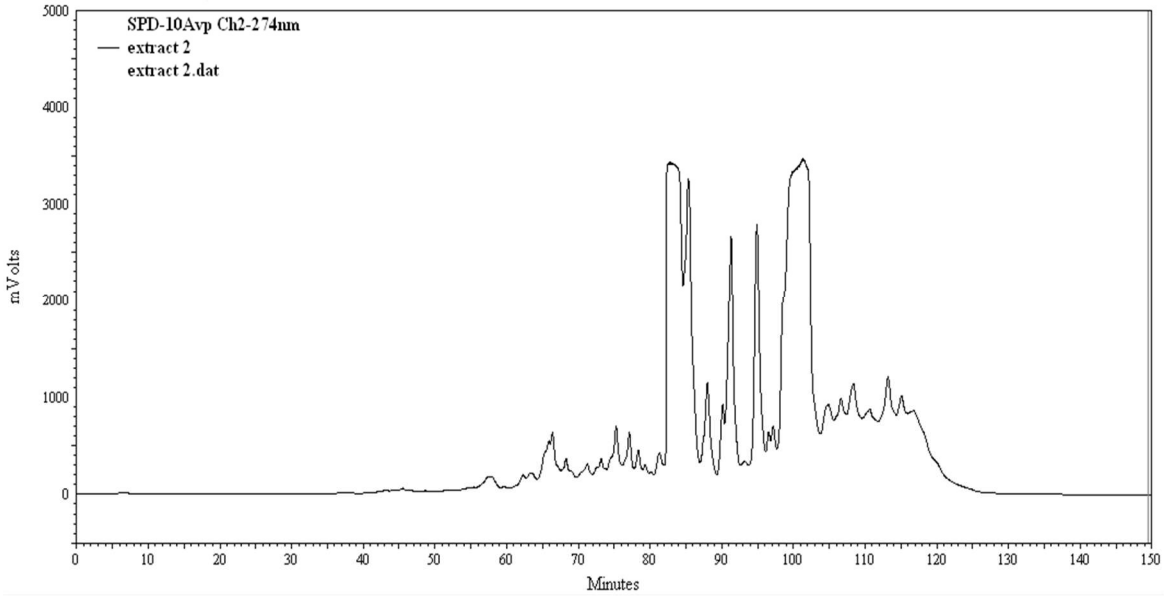


Figure 23: Chromatogram of CBDA extract from Soxhlet extraction using the preparatory HPLC method with Gradient 2.

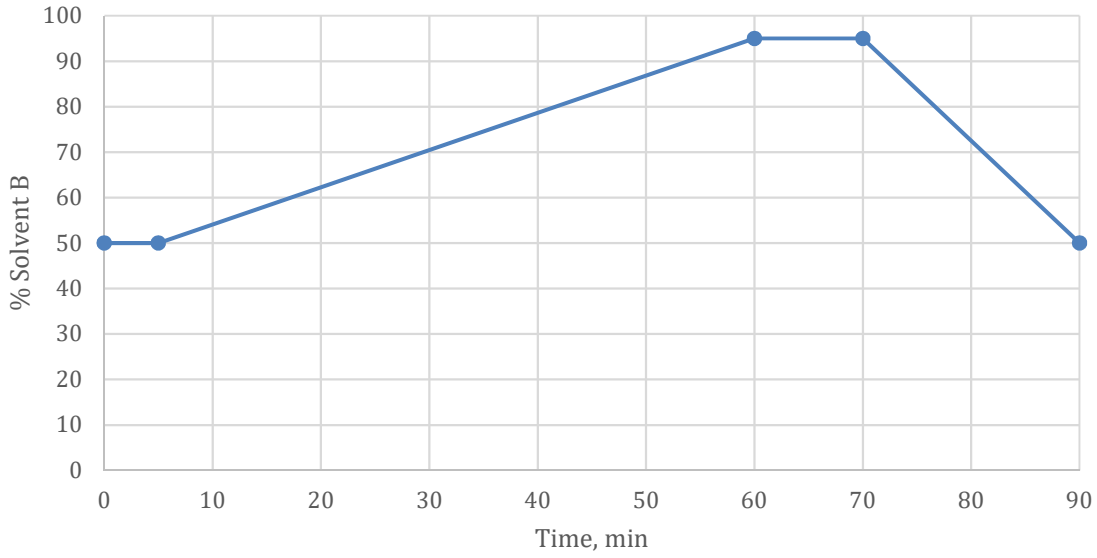


Figure 24: Gradient Method 3

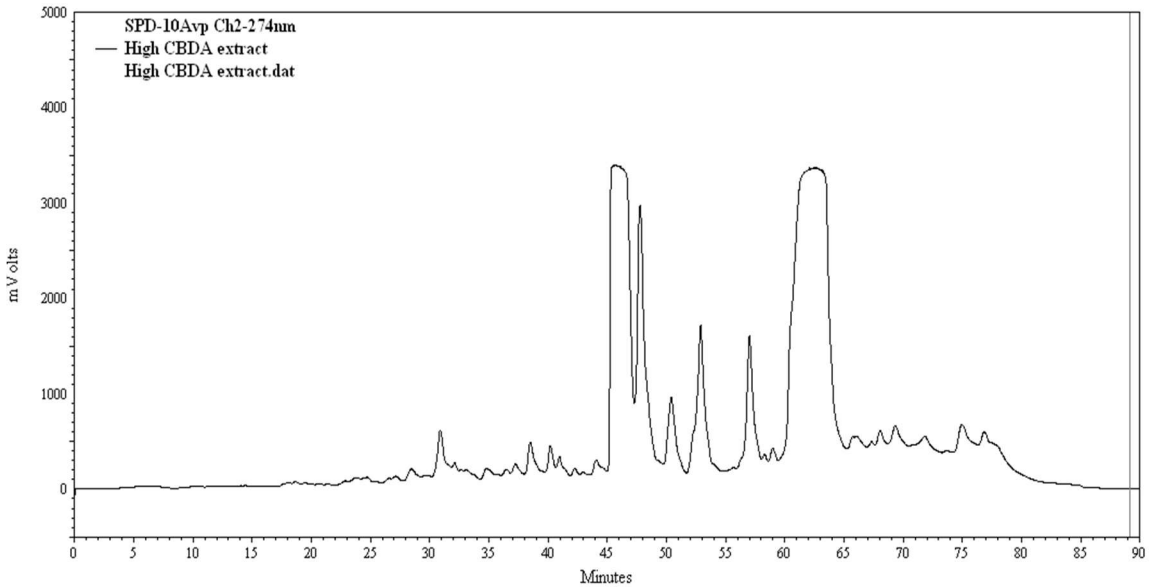


Figure 25: Chromatogram of CBDA extract the from Soxhlet extraction using the preparatory HPLC method with Gradient 3.

When the sample concentration exceeds the range of the detector, the detector cannot generate a larger response, and typically a flat-topped peak is observed, referred to as detector overload (Dolan, 2015). Two flat-topped peaks in the chromatogram were observed, so to address this issue the extract was diluted with two successive 1:2 dilutions with absolute ethanol prior to injection on the HPLC which was sufficiently dilute to prevent detector overload (Figure 26). This dilution factor also allowed for only the seven most abundant cannabinoids to be detected.

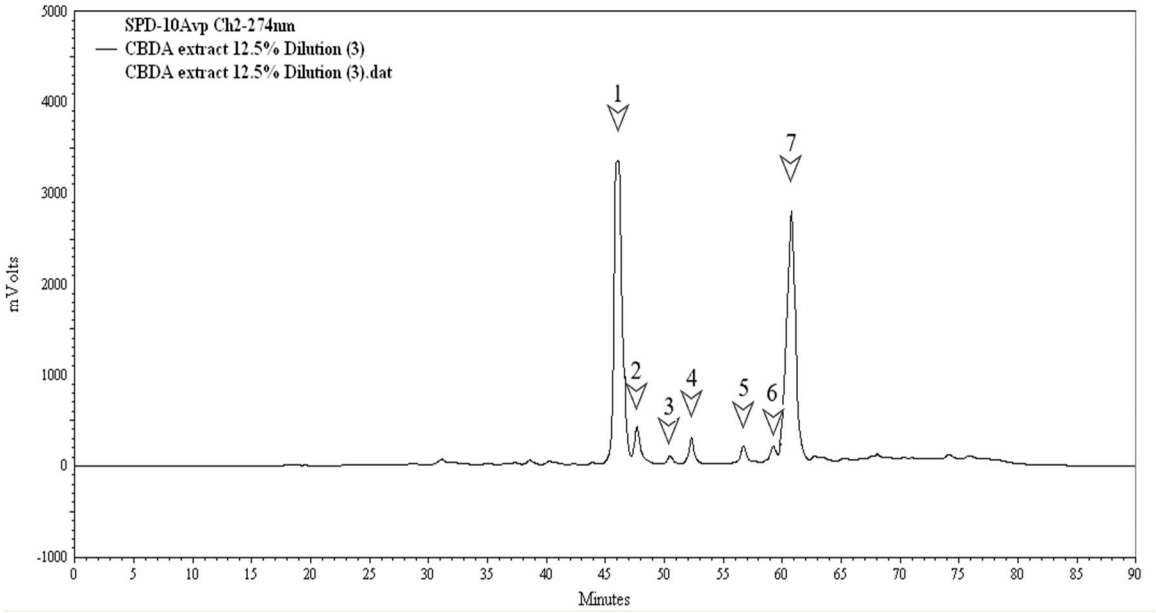


Figure 26: Chromatogram of CBDA extract, after two successive 1:2 dilutions with absolute ethanol from the Soxhlet extraction using Gradient 3.

Chromatographic Characterization of Cannabidiolic Acid

Once the preparative HPLC method was optimized, fractions were collected every minute to isolate each individual peak for analysis. Thirty test tubes of 4.5 mL of eluent were collected in total. Of the thirty, nine test tubes contained compounds of interest which corresponded to seven peaks on the chromatogram and these were collected for

analysis. A summary of fractions collected is given in Table 3. Fractions were prepared for analysis by combining test tubes which contained eluent from the same peak. The acetonitrile was removed by rotary evaporation and the cannabinoids were extracted from the remaining ammonium formate solution with 5 mL of pentane. The pentane was then evaporated to dryness with nitrogen gas; the resulting residue was resuspended in 1.0 mL absolute ethanol and analyzed by the analytical HPLC method.

Table 3: Summary of Collected Fractions from the Preparatory HPLC Method.

Collection Time	Fraction Number	Peak Number (Figure 26)	Peak Area	Retention Time (min.)	Identity
40-44	1-5	N/A	-	-	-
45-46	6-7	1	251815642	46.025	CBDA
47	8	2	53227248	47.683	Unknown
48-49	9-10	N/A	-	-	-
50	11	3	75508734	50.492	Unknown
51	12	N/A	-	-	-
52	13	4	16942665	52.300	Unknown
53-55	14-16	N/A	-	-	-
56	17	5	17919678	56.700	Unknown
57-58	18, 19	N/A	-	-	-
59	20	6	17791656	59.233	Unknown
60-61	21, 22	7	294579036	60.758	THCA
62-70	23-30	N/A	-	-	-

The most abundant cannabinoids of *Cannabis sativa* are Δ^9-THCA and CBDA (Elsohly & Slade, 2005). The area under the curve of peak 1 and peak 7 accounted for more than 55% of all compounds detected, making up 29.92% and 25.58 % of the area, respectively as reported in the generated area report. Because peak 1 and 7 (Figure 26) were the most abundant, this suggested that the identity of the compounds which produced these peaks may be CBDA, the analyte of interest, and Δ^9-THCA, the other most abundant cannabinoid in the extracted *Cannabis* sample. The compound from the

first major peak, collected from fractions 6 and 7, had a retention time of 46.025 minutes using the preparatory HPLC method. When analyzed with the analytical HPLC method, the compound had a retention time of 7.0 minutes (Figure 27) which matched the retention time for the standard CBDA (Figure 28). This was the first indication that CBDA had been successfully recovered from the Soxhlet extraction of the *Cannabis* plant material followed by the preparatory HPLC method. The identity of the compound which produced peak 1 (Figure 26) as CBDA was further supported by NMR and IR spectroscopy.

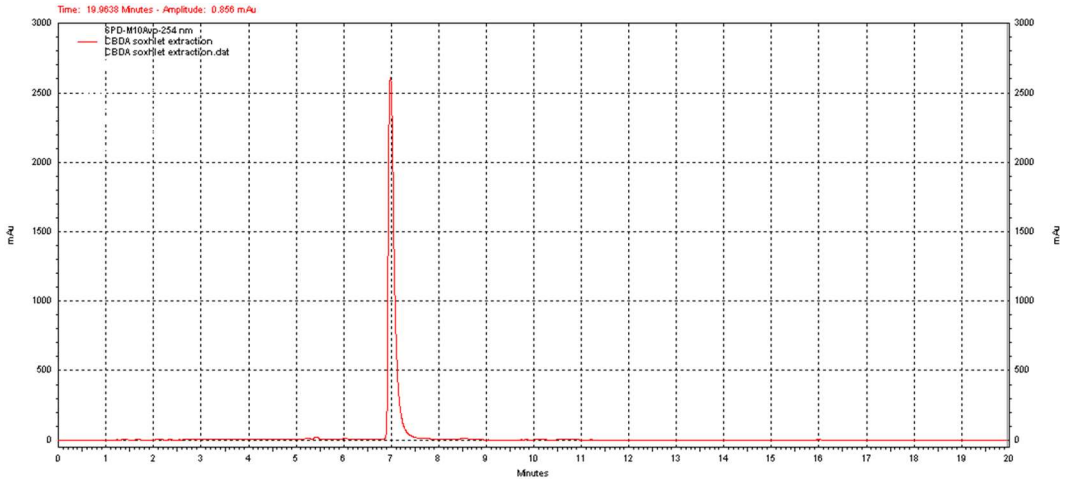


Figure 27: Chromatogram using the analytical HPLC method of the compound recovered from peak 1. Retention time 7.0 minutes.

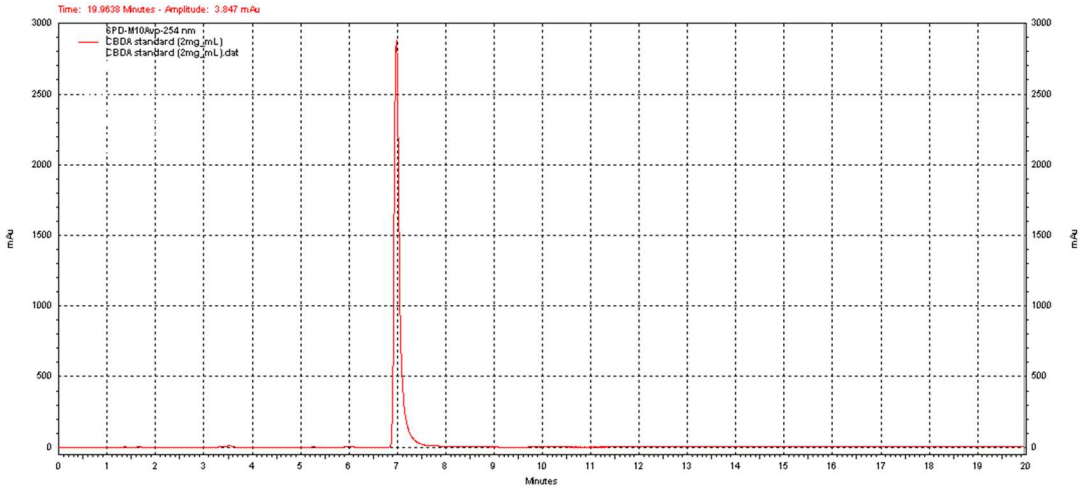


Figure 28: CBDA standard (2.0 mg/mL) using the analytical HPLC method. Retention time 7.0 minutes.

Nuclear Magnetic Resonance
Spectral Analysis

Both ^{1}H-NMR and ^{13}C-NMR spectra of the compound from peak 1 were obtained for comparison to spectra reported in the literature by Choi et al. (2004) and by Hazekamp, Choi and Verpoorte (2004). The study by Choi et al. (2004), which studied Δ^{9}-THCA, THC, Δ^{8}-THC, CBG, CBN, CBDA, CBD, and two flavonoids, was used for overall assignment of each peak in the ^{1}H-NMR and ^{13}C-NMR spectra. Unique singlets in the range of δ 4.0—7.0 in the ^{1}H-NMR spectrum of Δ^{9}-THCA, THC, CBDA, CBD, and CBN reported by Hazekamp et al., were used to further support the identity of CBDA once the spectrum was fully assigned. The numbering scheme for cannabidiolic acid used in these two studies is given in Figure 29. Although the spectra for cannabidiolic acid from the Choi et al. study were not provided, they reported a table of chemical shifts (ppm) with reference to TMS. Table 7 compares actual values from the ^{1}H- and ^{13}C-NMR obtained for the compound in peak 1 with values reported in the Choi et al. study for cannabidiolic acid.

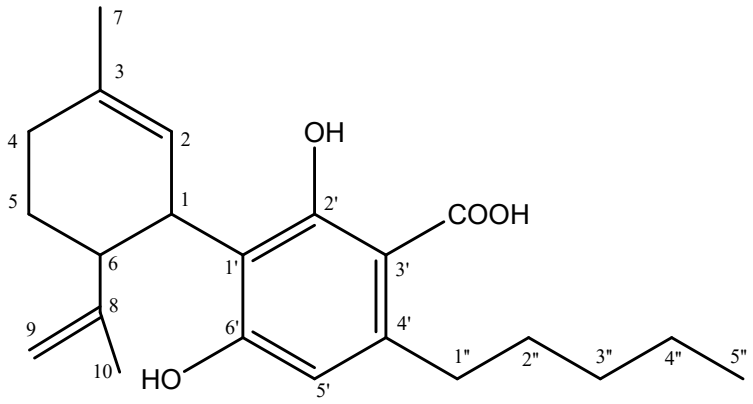


Figure 29: Numbering scheme used by Choi et al. (2004) for CBDA.

Table 4: Comparison of ^{1}H- and ^{13}C-NMR Spectra of the Compound Isolated from Peak 1 to Assignments for Cannabidiolic Acid Reported by Choi et al. (2004).

	^{1}H NMR		^{13}C NMR	
Position	ppm (actual, peak 1)	ppm (literature)	ppm (actual)	ppm (literature)
1	4.09	4.09	35.36	37.3
2	5.57	5.56	123.84	126.9
3			140.55	133
4	2.23	2.2	30.17	31.7
5	*	1.86	27.7	30.8
6	2.38	2.4	46.6	45.7
7	1.8	1.8	23.7	23.6
8			147.8	150.4
9	4.54	4.55	111.34	110.5
	4.39	4.4		
10	1.72	1.72		
1'			18.86	19.3
2'			114.45	116.5
3'			164.1	161.8
4'			102.5	104.2
5'	6.26	6.26	147.1	147
6'			111.9	111.3
1"	2.92, 2.82	2.92, 2.82	161	150.4
2"	1.58	1.57	36.56	37.6
3"	1.33	1.33	31.2	32.8
4"	1.33	1.33	31.97	33.2
5"	0.89	0.89	22.54	23.5
2'-OH	11.85	11.93	14.07	14.4
6'-OH	6.65	6.63		
COOH			176.06	175.6

*peak is absent in the spectra, assumed to be buried beneath the signal at 1.8 ppm

In the ^{13}C-NMR spectrum of the isolated compound (Figure 30), twenty-two distinct carbon peaks were observed as would be expected for cannabidiolic acid ($C_{22}H_{30}O_4$) (Figure 29). Choi et al. reported only twenty-one distinct peaks, noting that carbons in position 8 and 6' overlapped and appear as one signal at 150.4 ppm. Differences in chemical shifts from those reported in the literature could be attributed to the fact that Choi et al. obtained ^{13}C NMR spectra at 100 MHz in deuterated methanol, whereas the spectra obtained in this study were obtained at 100 MHz in deuterated

chloroform making it possible for the electronic environment of the atoms in these solutions to be different due to solute-solvent interactions.

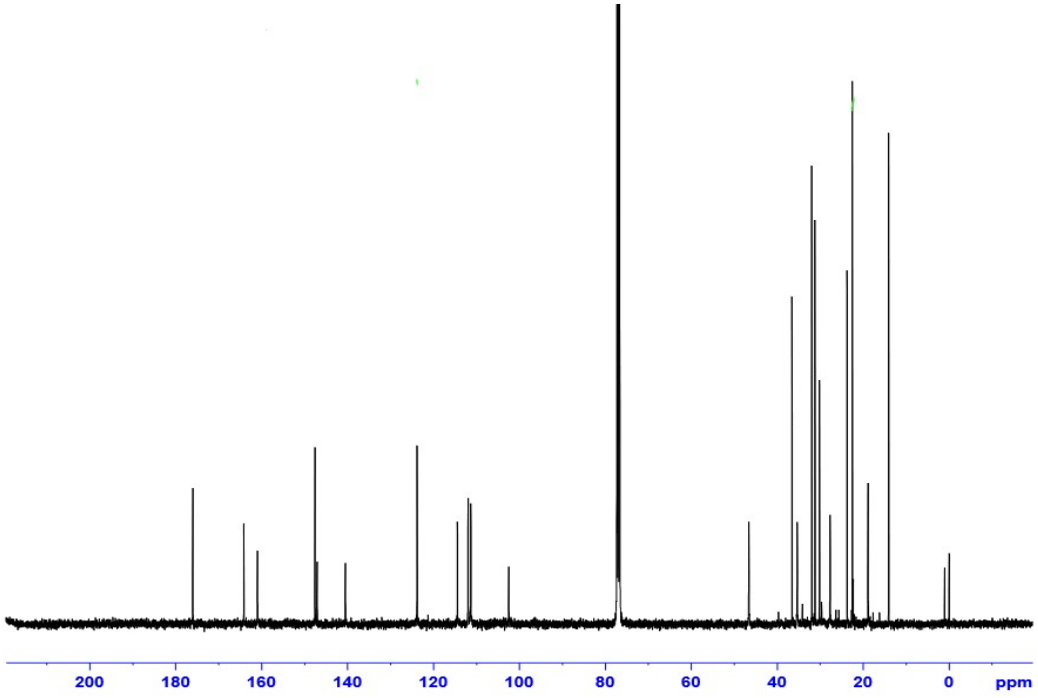


Figure 30: ^{13}C-NMR spectrum of the compound isolated from peak 1 in $CDCl_3$.

Unlike the ^{13}C-NMR, conditions for obtaining ^{1}H-NMR spectra were the same in both the literature references and in this study. As shown in Figure 31, a carboxylic acid proton is observed at 11.858 ppm, as would be expected from a carboxylic acid proton. Choi et al. reported the proton signal at 11.93 is from the 6' -OH but did not report a carboxylic acid hydrogen which typically is observed between 10-13 ppm. Phenolic hydrogens with intramolecular hydrogen bonding have a much broader range (10.5-16 ppm) (Jackman and Sternhell, 1969), thus it is possible that the signal is indeed from the 6'-OH and that the carboxylic acid hydrogen is absent. A carboxylic acid carbon signal can be observed, however, in the ^{13}C spectrum at 176 ppm, thus it can be confirmed that

the carboxylic acid carbon is present. The H-5 signal was not observed in the spectrum for the compound from peak 1, however the H-7 peak at 1.80 ppm integrated for six hydrogens when it should integrate for three hydrogens. The H-5 signal that is supposed to be present at 1.86 ppm could be buried beneath this peak, accounting for two hydrogens, suggesting that the last hydrogen which was unaccounted for in the integration to be the 6'-OH hydrogen.

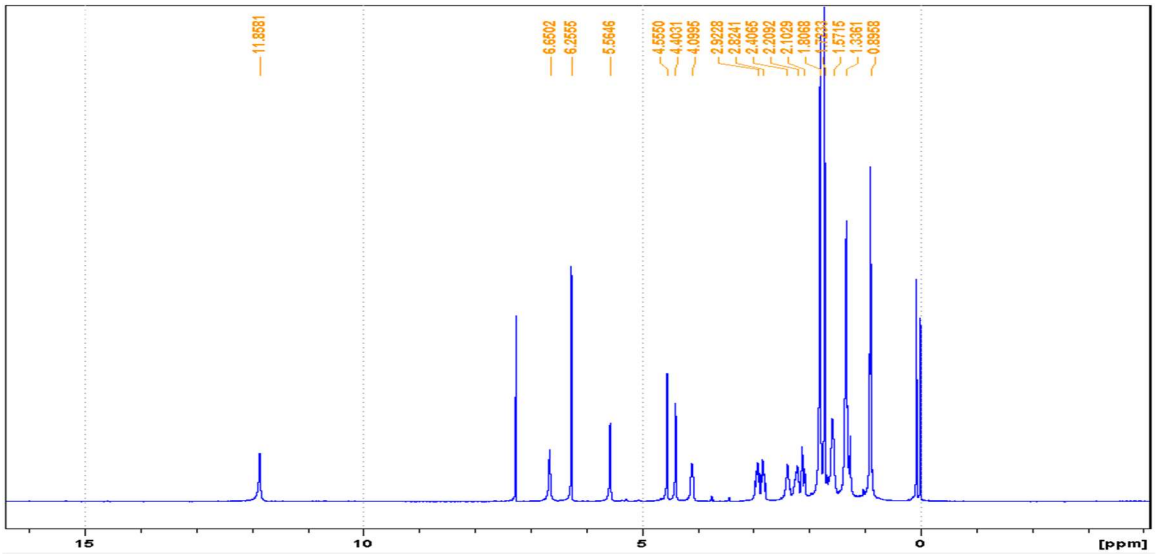


Figure 31: ^{1}H-NMR spectrum of compound isolated from peak 1 in $CDCl_3$

Because the disputed signals are from acidic hydrogens in CBDA, a technique for confirming signals from exchangeable protons called a deuterium exchange was performed by adding 100 µL of D_2O directly into the NMR tube containing the sample. Spectra of the CBDA isolated from the preparatory column before (A) and after (B) the addition of D_2O are presented in Figure 32. The absence of the signals at 11.85 and 6.65 ppm in the spectrum after deuterium exchange (Fig. 32 B) confirms that those two signals came from exchangeable hydrogens. No additional information was gained as to the assignments of the carboxylic acid -OH, 6'-OH, and 2'-OH. In order to further validate that the compound was in fact CBDA, signals in the 4.0-7.0 region where distinguishable

singlets between cannabinoids can be observed were compared. Four distinct signals for H-4, H-10, H-9 *trans* and H-10 *cis* are reported at 6.26, 5.55, 4.54, and 4.40 in the literature (Hazekamp et al., 2004) and observed in the spectrum obtained.

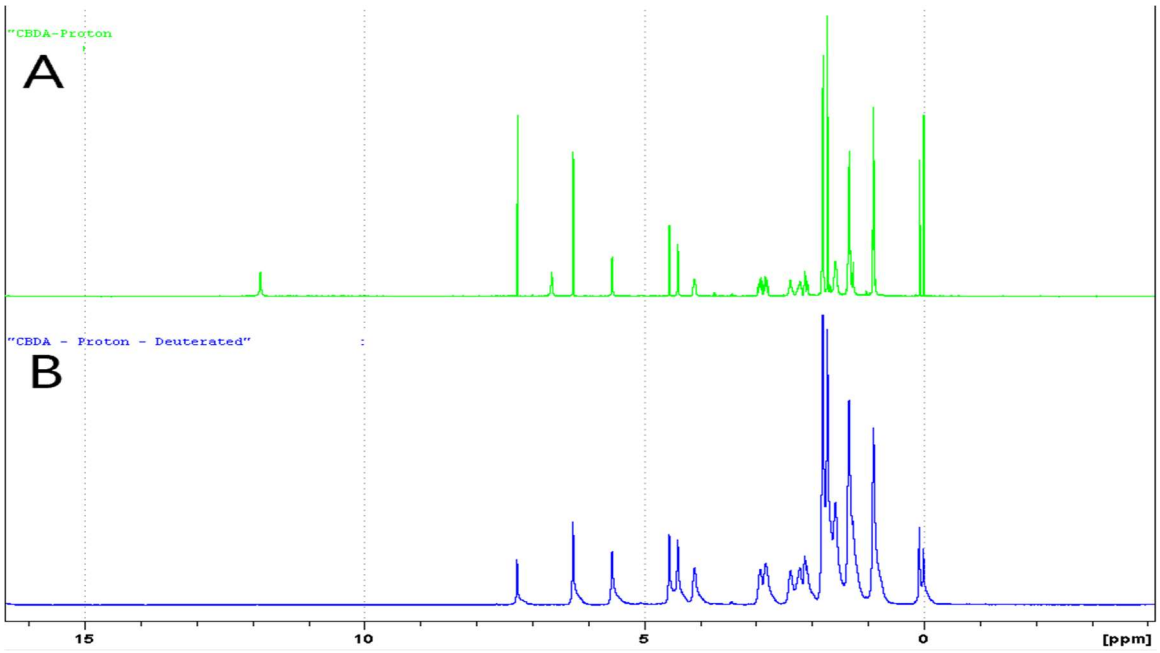


Figure 32: Deuterium exchange experiment for the compound isolated from peak 1. (A) before the addition of D_2O and (B) after the addition of D_2O.

Infared Spectral Analysis

Additional spectral analysis included infrared spectroscopy which was obtained from a viscous 1.0 mg sample of the compound isolated from peak 1. A broad peak in the IR spectrum, starting at approximately 3400 cm^{-1}, can be observed (Figure 33) as well as a sharp peak at 1617.30 cm^{-1} and a sharp peak at 1257. 32 cm^{-1}. These three signals together are indicative of a carboxylic acid -OH and its C=O and C-O stretches. Comparing this spectrum to the IR spectrum by Hazekamp et al. (2005), both have a broad carboxylic acid peak, along with sharp peaks at approximately 1250 cm^{-1}. The peaks protruding around 2900 cm^{-1} match C-H stretched indicated by Hazekamp et al.

This spectrum provided further confirmation that the identity of the compound present in peak 1 is CBDA.

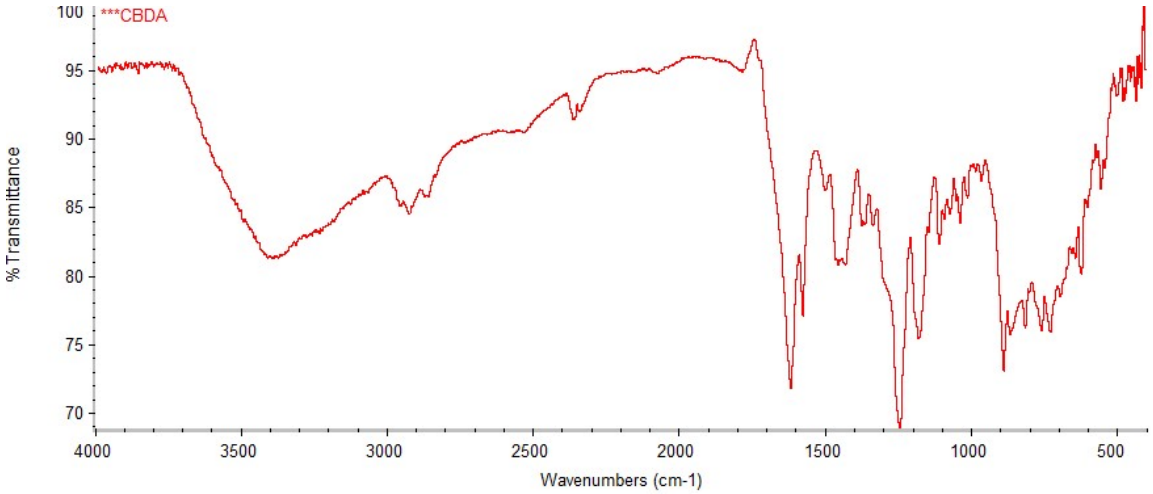


Figure 33: IR analysis of the compound isolated from peak 1.

Recovery

The *Cannabis* plant material contained a reported 6.5% CBDA. To determine the perecnt of CBDA recovered by preparatory HPLC following Soxhlet extraction, a 0.5 mL sample of pure extract was separated by preparatory HPLC, collected by the fractionator, and processed with rotary evaporation and back-extraction into pentane. The pentane was evaporated in a pre-weighed container and the mass of the recovered CBDA was detemined for the 0.5 mL injection volume. This mass was multiplied by 32 to account for the total volume of 16 mL for the extract. On average, 77.4 % of the repored CBDA was recovered from the 0.325 g expected from 5.00 g plant material . Table 5 summarizes recovery data.

Table 5: Percent Recovery Data

Injection	CBDA Recovered* (g)	Calculated total CBDA extracted (g)	% CBDA Recovered
1	0.00755	0.2416	74.34
2	0.00841	0.2691	82.81
3	0.00697	0.2440	75.08

*Recovered from 0.5 mL injection of pure extract

Other Cannabinoid Analyses

Although CBDA was the focus of this study, this preparatory method could potentially be used to separate a variety of compounds extracted from *Cannabis* plant material. The compound from the last major peak, peak 7, collected from fractions 21 and 22, had a retention time of 60.8 minutes on the preparatory column. When analyzed with the analytical HPLC method, the compound had a retention time of 10.8 minutes (Figure 34) which was comparable with a reference standard for THCA with a retention time of 10.6 min (Figure 35). When THCA is in a mixture of cannabinoids, it appears to elute slightly later than when in an pure solution, similar to the effect observed when CBDA and CBD are in a mixture.

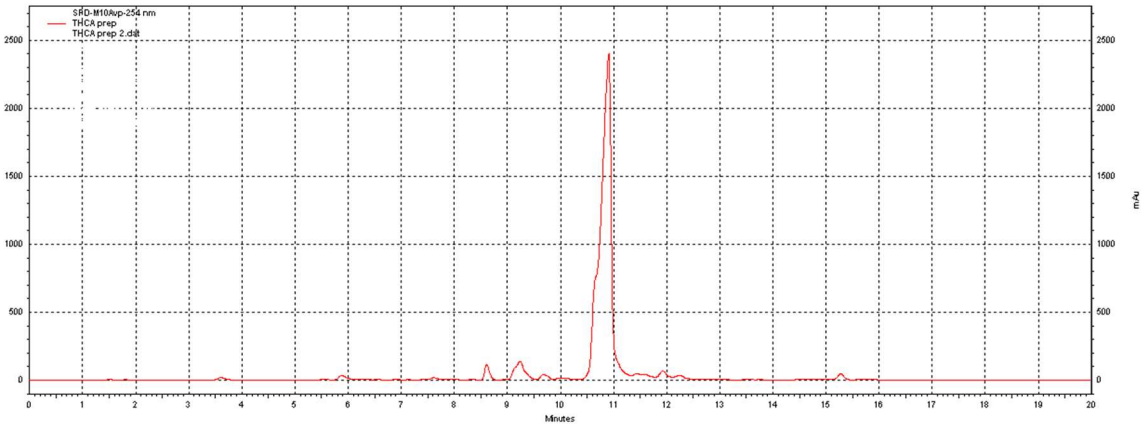


Figure 34: Chromatogram using the analytical HPLC method of the compound recovered from peak 7 using the preparatory HPLC method. Retention time 10.8 minutes.

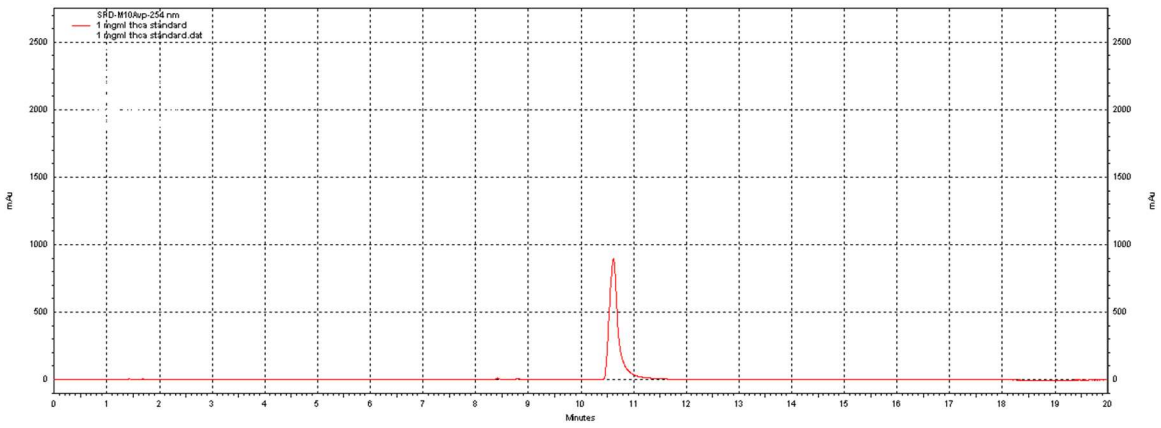


Figure 35: Chromatogram of THCA standard (1.0 mg/mL). Retention time 10.6 minutes

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In an effort to identify which other cannabinoids were present, an additonal preparatory run was performed but instead of collecting individual fractions all compounds which eluted between 40 and 70 minutes were collected. This fraction was prepared for analysis and chromatographed on the analytical column (Figure 36), which was compared to the phytocannabinoid standard mixture (Figure 37) that contained 100 µg/mL of each of the following cannabinoids: CBDA, cannabigerolic acid (CBGA), cannabigerol (CBG), CBD, tetrahydrocannabivarin (THCV), cannabinol (CBN), Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA), Δ^9-tetrahydrocannabinol (Δ^9-THC), Δ^8-tetrahydrocannabinol (Δ^8-THC), and cannabichromene (CBC).

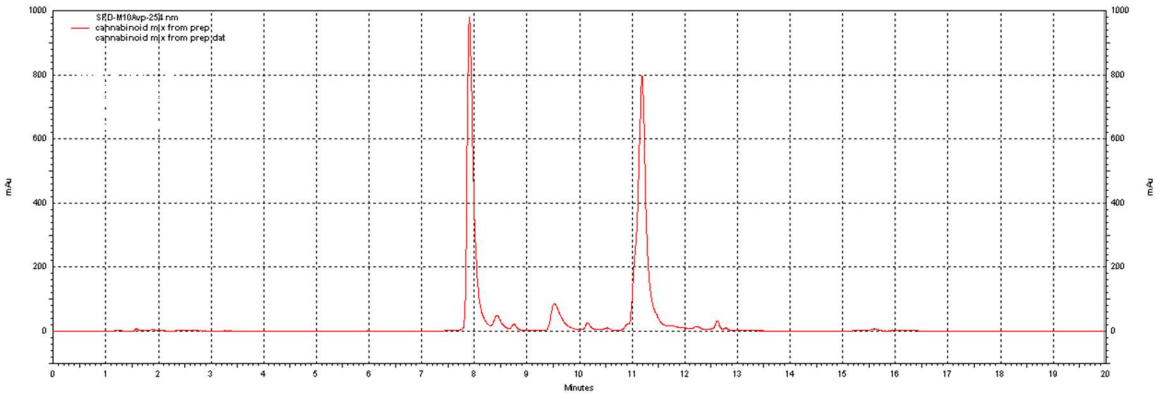

Figure 36: Chromatogram generated using the analytical HPLC method of the cannabinoid mixture recovered from the 40-70 minute fraction using the preparatory HPLC method with Gradient 3.

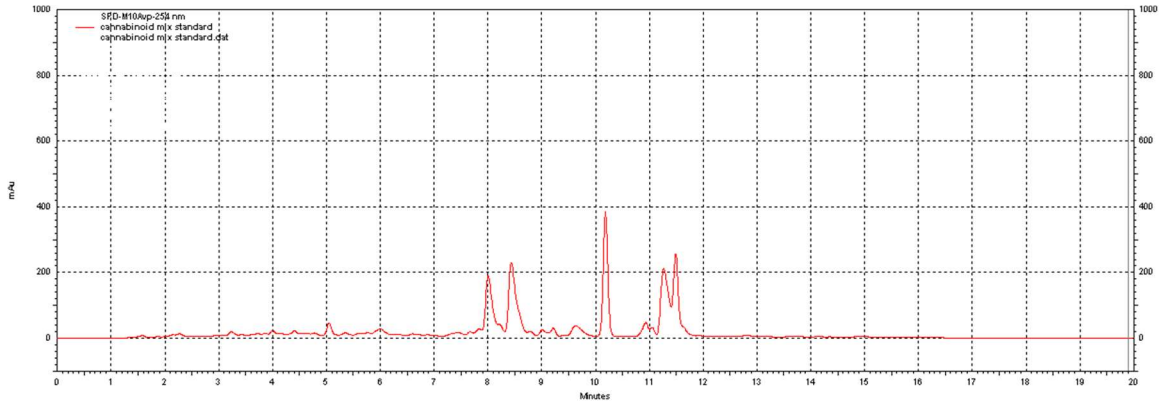

Figure 37: Chromatogram of phytocannabinoid mixture standard using the analytical HPLC method.

It appears that, like CBDA and CBD, other cannabinoids will elute at different times in a mixture possibly due to interactions between compounds than when in a pure sample. It was assumed that the peak at 7.9 minutes (Figure 36) is CBDA and the peak at 11.3 minutes is THCA due to abundance and order of elution. It appears that the crude extract contains several of the standard cannabinoids: one which elutes at 8.4 min., the next at 8.9 min., followed by one at 9.6 min. and one which elutes at 10.1 min. Each fraction was analyzed with the analytical HPLC method and chromatograms were collected (Appendix B). These chromatograms indicated that each sample was impure and therefore not suitable for further analysis to confirm the identity. If pure cannabinoid standards were obtained, other than the major cannabinoids, more studies could follow to identify which of the standards match the compounds in this crude extract; however, this was outside the scope of this study.

Cell Assay for Decarboxylase Activity

To identify a microbial species which exhibits decarboxylase activity, seven cell lines were screened, four bacterial and three fungal. Species were chosen based upon

literature reports of decarboxylase activity for compounds similar in structure to CBDA. Species were screened using cells which were either intact or lysed and either induced or uninduced depending on the assay. A summary of cell assays is given in Table 6. A full description of the various combinations of conditions for each assay is given in Table 7 (Appendix A).

Table 6: Summary of Cell Incubations for Decarboxylase Activity.

Cell Type	Intact	Lysed	Induced	Induction Substrate
Aspergillus niger	No	Yes	Yes	Salicylic acid
Aspergillus clavatus	No	Yes	Yes	Salicylic acid
Bacillus subtilis	Yes	Yes	Yes	Salicylic acid or 4-hydroxybenzoic acid
Enterobacter (Klebsiella) aerogenes	Yes	Yes	No	Not induced
Klebsiella pneumonia	Yes	Yes	Yes	2,4-dihydroxybenzoic acid
Penicillium chrysogenum	No	Yes	Yes	Salicylic acid
Pseudomonas putida	Yes	Yes	Yes	Salicylic acid or 2,4-dihydroxybenzoic acid

From the bacteria screened, *B. subtilis, E. aerogenes,* and *K. pneumonia* displayed no detectable CBDA decarboxylase activity; representative chromatograms for each species are given in Appendix C1, C2, and C3, respectively. *B. subtilis* was assayed under various combinations of intact/lysed and uninduced/induced conditions, however no CBDA decarboxylase activity was observed. This cell line was not tested with model compounds but was chosen because it expresses the genes for both a phenolic acid decarboxylase and a phenylacrylic acid decarboxylase. Duy et al. (2007) showed that these enzymes were both expressed when induced with salicylic acid. However, even

after induction with salicylic acid no CBDA decarboxylase activity was observed in the current study when *B. subtilis* was incubated in the presence of CBDA.

E. aerogenes contains an enzyme which decarboxylates both Category 1 and 2 substrates, and this cell line was assayed both intact and lysed. Earlier work by Cribbs (2014) demonstrated the ability of lysed *E. aerogenes* cells to decarboxylate the Category 2 substrate p-hydroxybenzoic acid but not any phenolic carboxylic acid with hydroxyl groups *ortho* to the carboxylic acid. A permeability barrier for the substrate of the decarboxylase was noted in the study by Grant and Patel (1969) which could explain the lack of activity in intact cells. An optimal pH of 6 was also noted which could explain a lack of detectable activity when incubated in the presence of CBDA, even with lysed cells, because all assays were performed at the pH of the media.

Both intact and lysed *K. pneumonia* cells, induced with 2,4-dihydroxybenzoic acid, were incubated in the presence of CBDA. No decarboxylase activity was observed in any of the three assays performed. Like *E. aerogenes,* the decarboxylase enzyme from *K. pneumonia* is able to decarboxylate the Category 2 substrate p-hydroxybenzoic but no model compound with a phenolic hydroxyl group *ortho* to the carboxylic acid (Cribbs, 2014).

None of the fungal species screened displayed CBDA decarboxylase activity when incubated in the presence of CBDA. Representative chromatograms for assays of *A. clavatus, A. niger,* and *P. chrysogenum* are given in Appendix C4, C5, and C6, respectively. *Aspergillus clavatus* was not tested with model compounds but was chosen because it has the *patG* gene, which codes for an enzyme that decarboxylates 6-methylsalicylic acid. Two assays of *Aspergillus clavatus* induced with salicylic acid and

one assay of *A. clavatus* induced with CBDA were performed but no CBDA decarboxylase activity was observed. *Penicillium chrysogenum* and *Aspergillus niger* did not demonstrate any decarboxylase activity when incubated with model compounds (Cribbs, 2014) or with CBDA in the current study.

In previous studies, *Pseudomonas putida* was demonstrated to catalyze the decarboxylation of 2-hydroxy-4-methoxybenzoic acid (Cribbs, 2014). In the current study, twelve assays were performed for CBDA decarboxylase activity with *P. putida.* A representative set of chromatograms are presented in Figures 38-42. Individual treatments for each assay are given in Table 7, (Appendix A) and additional representative chromatograms are given in Appendix C7. CBDA typically elutes at 7.0 minutes with the analytical HPLC method; however the column oven was not set, and samples were run at ambient room temperature for this assay, resulting in retention times of the standards for CBDA and CBD 7.8 and 8.3 minutes, respectively. Figure 38 shows a chromatogram of an incubation extraction at time 0 with only one major peak, CBDA at 7.8 minutes. After 1 hour, CBDA was still the only species present (Figure 39). After 2 hours of incubation, the peak for CBDA was still be observed, and a peak for CBD appeared at 8.3 minutes (Figure 40). The observation of both peaks persists at time 3 hr. (Figure 41) and 4 hr. (Figure 42), with a slight increase in peak intensity in the final hour, t = 4 hr., of assay.

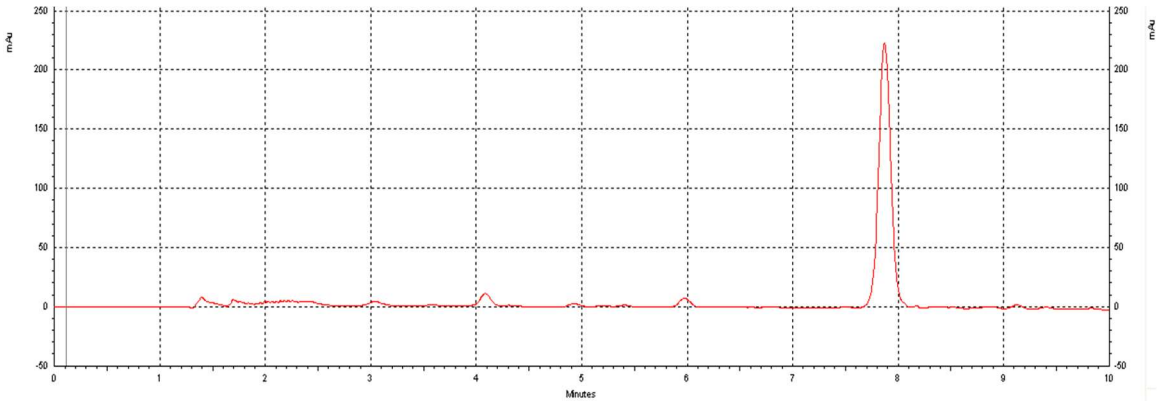

Figure 38: Chromatogram of CBDA after 0 hours of incubation with lysed *P. putida*. CBDA is observed at 7.8 min.

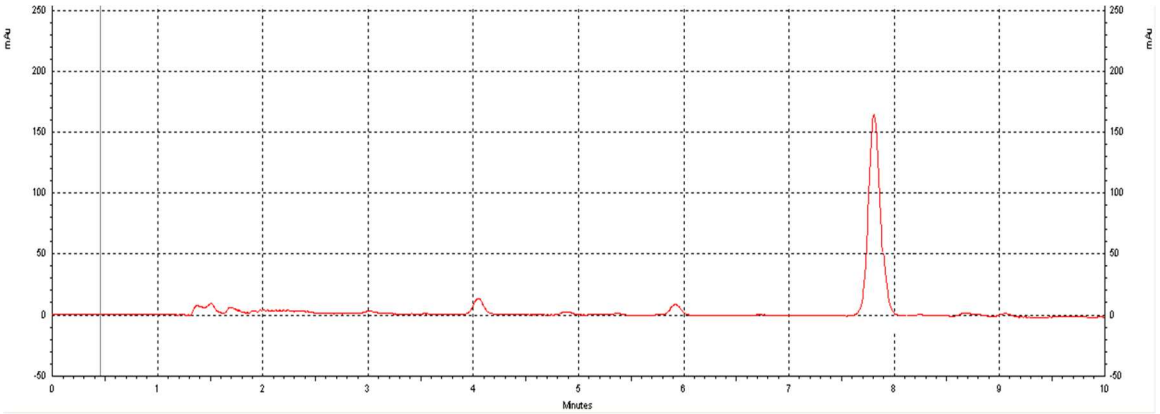

Figure 39: Chromatogram of CBDA after 1 hour of incubation with lysed *P. putida.* CBDA is observed at 7.8 min.

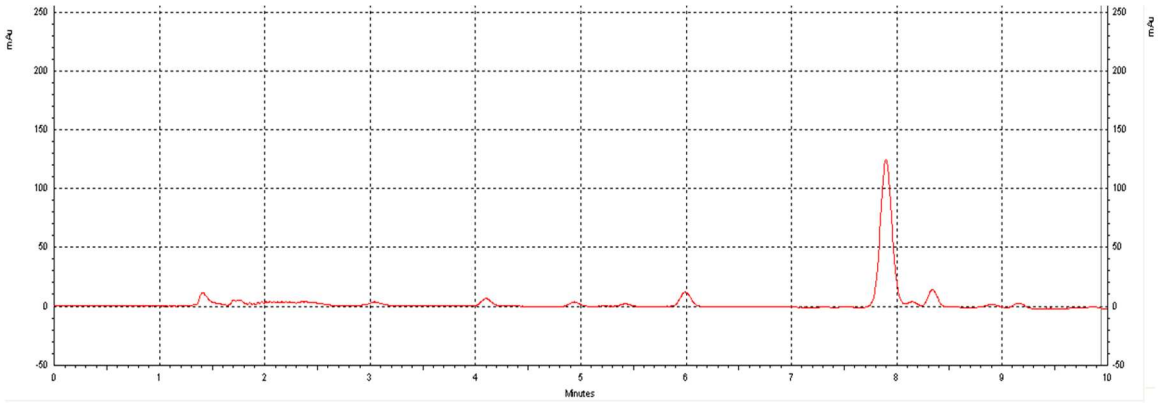

Figure 40: Chromatogram of CBDA after 2 hours of incubation with lysed *P. putida*. CBDA (7.8 min) and CBD (8.3 min) are observed

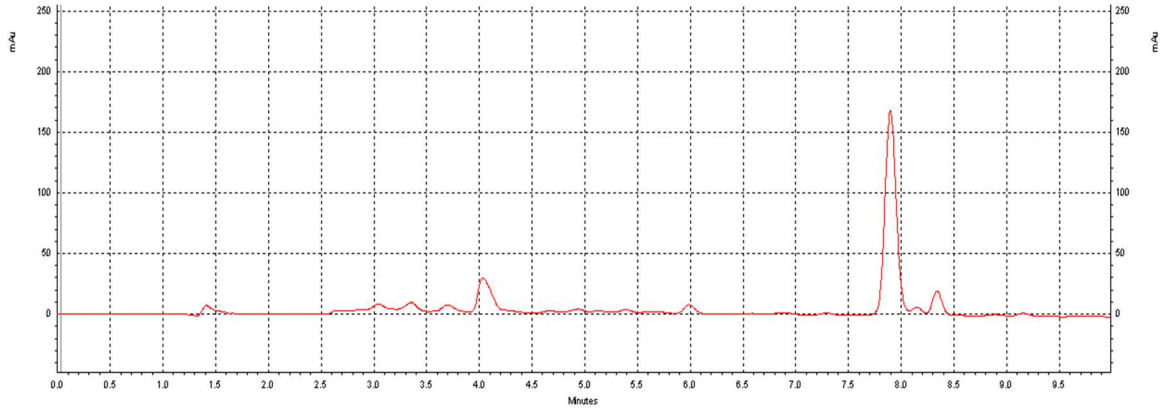

Figure 41: Chromatogram of CBDA after 3 hours of incubation with lysed *P. putida*. CBDA (7.8 min) and CBD (8.3 min) are observed.

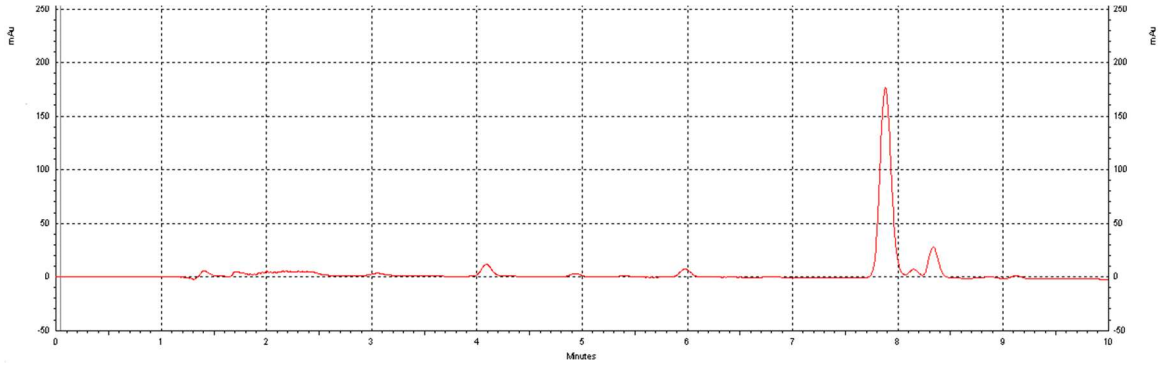

Figure 42: Chromatogram of CBDA after 4 hours of incubation with lysed *P. putida*. CBDA (7.8) and CBD (8.3) are observed.

An autoclaved sample of the *P. putida* used as a control was assessed concurrently with the active cell culture to be sure any observed conversion was, in fact, produced by an enzyme present in the cell. Only CBDA was observed for all time points with the control. The observation of the appearance of CBD suggests that *Pseudomonas putida* has an enzyme which can convert the prodrug CBDA into the physiologically active CBD. To calculate the rate at which this occurs, the area under the curve for CBD was taken and calculations were performed in Excel using the volume of the injection (10 µL) and the molar mass of CBD (314.46 g/mol). The rate at which CBD appeared was determined to be 5.0 nmol/hour.

CHAPTER V

CONCLUSIONS

The first goal of this research was to isolate and identify CBDA from an extract prepared in-house from *Cannabis* plant material. Isolating cannabinoids in-house from plant material has several benefits for this research. The plant material itself is considerably less expensive relative to the cost of a pure, isolated cannabinoid. Reagent cost and a minimal on-hand quantity of the desired cannabinoid can hinder productive research, thus having the ability to isolate CBDA lowers cost and increases efficiency. In-house isolation also allows a more "green" approach because one is not required to use extra resources to ship reagents and can monitor quality control.

A Soxhlet extraction was performed to obtain an extract which was high in CBDA. The extract was then purified using a preparative HPLC method developed for the separation of cannabinoids. The CBDA obtained from this method was seemingly pure, as no other peaks were observed in the analytical HPLC chromatogram obtained and few unidentifiable peaks were observed in the ^{1}H- and ^{13}C- NMR spectra. Other cannabinoids could possibly be isolated with this method as well, however, CBDA was the only pure cannabinoid isolated in this study. The development of this method will allow further research to be conducted in our laboratory on various independently isolated cannabinoids.

The second goal of this research was to identify a cell line possessing enzymes capable of decarboxylating CBDA. In its physiologically active form, cannabidiol is supported by a vast body of literature as a potential cancer treatment. To accomplish this goal, several cell lines were screened for CBDA decarboxylase activity by monitoring conversion of CBDA to CBD via HPLC. An analytical HPLC method was successfully developed for this task. Cell lines were selected based on the literature and previous work in our research group with compounds which model cannabinoids (Cribbs, 2014).

The results indicate that a CBDA decarboxylase is present in *Pseudomonas putida*. Cultures of *P. putida* which were not induced or induced with 2,4-dihydroxybenzoate did not show any CBDA decarboxylase activity. However, cultures of *P. putida* that were induced with salicylic acid for three days and lysed prior to decarboxylase activity screening indicate the formation of CBD when incubated with CBDA for at least three hours. This decarboxylase activity from *P. putida* is the first enzymatic method for the decarboxylation of CBDA observed which does not require substantial heat to convert it to its physiologically active form CBD.

If a cell line was identified, therapeutic drug development can move forward using Cell-in-a-Box® technology. With the desired decarboxylation of CBDA to CBD observed, isolation of the gene for the decarboxylation enzyme would be the next logical step with cultures of *P. putida* which have been induced for three days with salicylic acid and lysed. The isolated gene could then be inserted into a human embryonic kidney (HEK) cell through genetic engineering. Once the HEK cell has been transfected with the decarboxylase gene, it can be analyzed with the same assay developed for microbial cells tested in this study, with the ultimate goal of encapsulating the HEK cells using

Cell-in-a-Box® technology and testing in clinical trials. This could be especially beneficial for patients with tumors which are difficult to remove surgically such as gliomas, which CBD has been shown to treat effectively. The specific activity should also be quantified because literature studies show that the effectiveness of CBD is dose-dependent.

Future Research

The preparatory HPLC method could be developed further to not only isolate CBDA but other major cannabinoids as well. The lack of purity of the other major cannabinoids collected in this study meant that obtaining NMR data would not prove useful for identification in this study. If the samples were pure, these could be collected and used to identify compounds other than CBDA and THCA. Current standards could also be spiked with standards to help identify unknown peaks in the current chromatograms.

The procedure for collecting fractions would have to be optimized in order to obtain compounds which are purer than those obtained in this study. This may be achieved by reducing the time increment for collection or by altering the preparatory HPLC parameters to achieve greater baseline separation between peaks. This could be achieved by using a different organic component to manipulate the polarity of the mobile phase. Acetonitrile could be substituted by a more polar solvent such as methanol which may increase run length but could provide better resolution of peaks. Methanol is also generally less expensive than acetonitrile with current prices from Sigma-Aldrich quoted at $69.50 and $223.00, respectively, for 1 L of HPLC-grade reagents.

The percent recovery of cannabidiolic acid could also be improved by minimizing transfer steps in the sample processing once collected from the HPLC. The current process requires evaporating the organic solvent and then back extracting from the aqueous phase into pentane. If the number of steps in this process were able to be reduced perhaps less CBDA would be lost in transfer and could increase the percent recovery.

Additional decarboxylase assays could be done to find additional microbial species capable of the decarboxylation of CBDA to CBD, with a focus on fungal species to determine if there might be a species which catalyzes this reaction more efficiently. The decarboxylase expressed by *A. clavatus* decarboxylates Category 1 substrates, those which are the most similar to the structure of CBDA; however, there was difficulty in homogenizing fungal samples when preparing them for assay. Work should be done with current collaborators who prepare cell cultures to optimize this method and then additional assays should be performed with cultures of different *Aspergillus* species such as *A. clavatus, A. giganteus, and A. longivesica* which were reported by Snini et al. (2013) to express the *PatG gene.* Another fungus which was not tested was *Trichosporon moniliform* which decarboxylates salicylic acid and many other carboxylic acids (Kirimura et al., 2010). Care needs to be taken in the initial stages of working with these types of cells since there is a risk of fungal infection. The risks associated with these types of cells should be mitigated by incorporating the gene for the decarboxylase in a genetically-engineered human embryonic kidney cell.

If additional decarboxylase assays are in order, a standard curve should be made in which CBDA and CBD are analyzed in tandem. This would assure that any effects

observed from interaction of each analyte with each other could be observed. This would also be a better application since they are not being analyzed individually in the context of the enzymatic assay. A standard curve which matches the application would be more beneficial than the current format of each individual standard curve.

Advancement in understanding of the therapeutic benefits of cannabinoids has propelled the scientific community forward to develop a multitude of cannabinoid-based therapies. A significant hurdle on the road to making the most beneficial medicine available to the patients has been delivering the appropriate dose of cannabinoids in a safe and effective matter. The results from this research provide an alternative to the traditional mode of activating cannabinoid pro-drugs and a new way to potentially control and monitor the effective dose of cannabinoids being administered.

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APPENDIX A

CONDITIONS FOR INDIVIDUAL CELL ASSAYS FOR DECARBOXYLASE ACTIVITY

Table 7: Conditions for Individual Cell Assays for Decarboxylase Activity

Species	Intact or lysed	Induced	Length of Induction	Wet Cell Mass (mg/mL)	Concentration of CBDA (mg/mL)*
Enterobacter (Klebsiella) aerogenes	intact	None	N/A	72.8	10.0
	lysed	None	N/A	72.8	10.0
Pseudomonas putida	intact	2,4-dihydroxybenzoate	1 day	40.4	10.0
	lysed	2,4-dihydroxybenzoate	3 days	40.4	10.0
	lysed	30 mM 2,4-dihydroxybenzoate	1 day	133	22.5
	lysed	30 mM 2,4-dihydroxybenzoate	3 days	69.5	20.0
	lysed	70 mM 2,4-dihydroxybenzoate	1 day	78	20.0
	lysed	10 mM salicylic acid	3 days	68.4	20.0
	lysed	10 mM salicylic acid	3 days	23.6	13.0
	lysed	10 mM salicylic acid	3 days	Not provided	11.0
	lysed	10 mM salicylic acid	3 days	65.5	16.5
	lysed	1 mM salicylic acid	3 days	54.2	2.0
	lysed	1 mM salicylic acid	3 days	58.5	2.0
	lysed	1 mM salicylic acid	3 days	22.3	2.0
Klebsiella pneumonia	intact	30 mM 2,4-dihydroxybenzoate	3 days	162	22.5
	intact	30 mM 2,4-dihydroxybenzoate	3 days	62.4	23.0
	lysed	30 mM 2,4-dihydroxybenzoate	3 days	74.4	23.0
	lysed	None	N/A	84	12.7
	lysed	CBDA	1 day	55.2	12.7
	lysed	30 mM 2,4-dihydroxybenzoate		42.4	15.0
Bacillus Subtilis	lysed	10 mM salicylic acid	2 days	308.8	23.0
	lysed	40 mM salicylic acid	2 days	46	23.0
	lysed	None	N/A	158	12.7
	lysed	CBDA	1 day	206.4	12.7
	lysed	None	N/A	78.1	10.0
	lysed	4 mM salicylic acid	3 days	52.2	10.0
Aspergillus clavatus	lysed	4 mM salicylic acid	3 days	Not provided	10.0
	lysed	4 mM salicylic acid	3 days	Not provided	10.0
Aspergillus niger	lysed	4 mM salicylic acid	3 days	Not provided	2.0
Penicillium chrysogenum	lysed	4 mM salicylic acid	3 days	Not provided	2.0
	lysed	4 mM salicylic acid	3 days	Not provided	13.5
	lysed	4 mM salicylic acid	3 days	Not provided	11.0

*Stock solution in absolute ethanol added to incubation in total of 100 µL.

APPENDIX B

CHROMATOGRAMS OF FRACTIONS 2-6 COLLECTED FROM THE PREPARATORY HPLC METHOD

Additional Chromatograms Using the Analytical HPLC Method

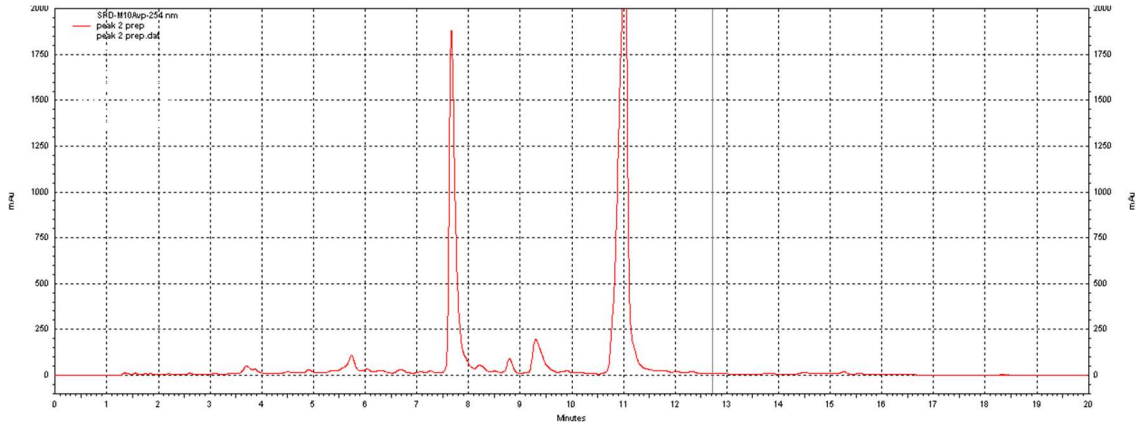

Figure 43: Chromatogram of the residue recovered representing peak 2 in the preparatory chromatogram. The sample was resuspended in 1.0 mL absolute ethanol and analyzed using the analytical HPLC method.

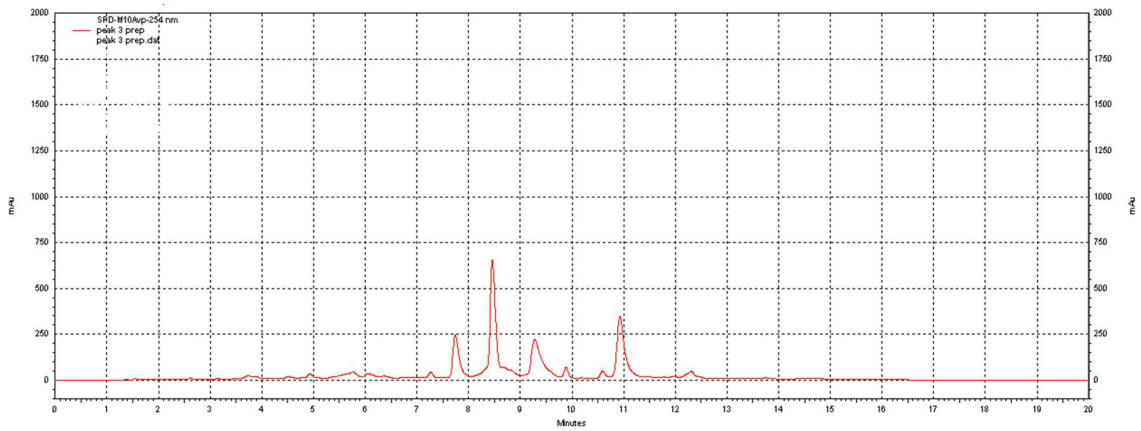

Figure 44: Chromatogram of the residue recovered representing peak 3 in the preparatory chromatogram. The sample was resuspended in 1.0 mL absolute ethanol and analyzed using the analytical HPLC method.

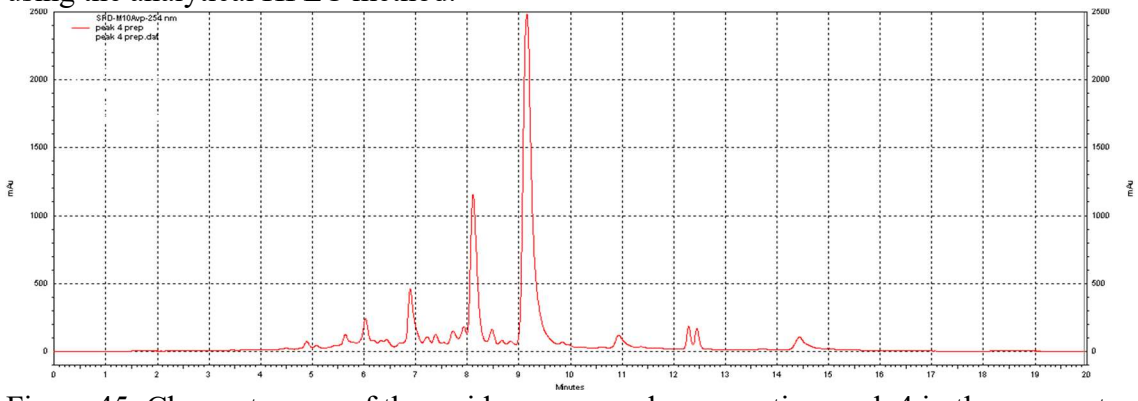

Figure 45: Chromatogram of the residue recovered representing peak 4 in the preparatory chromatogram. The sample was resuspended in 1.0 mL absolute ethanol and analyzed using the analytical HPLC method.

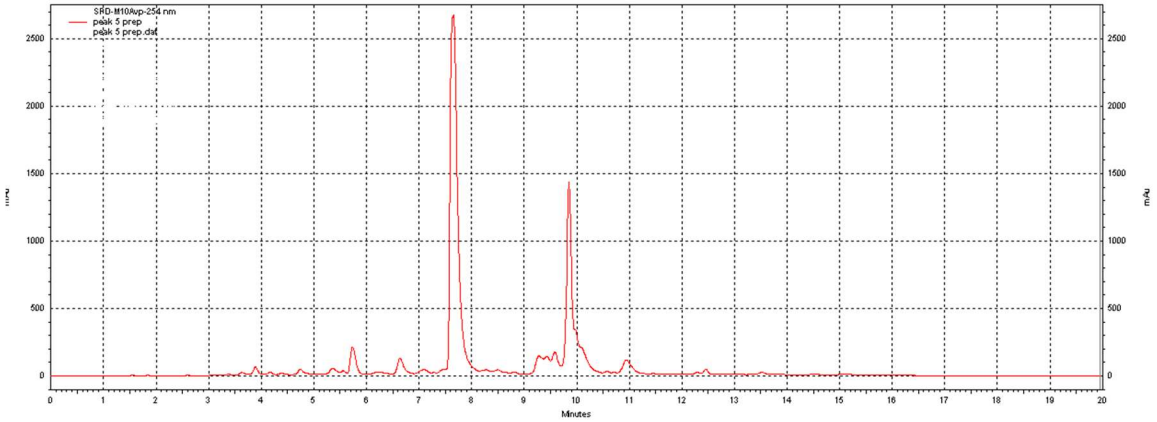

Figure 46: Chromatogram of the residue recovered representing peak 5 in the preparatory chromatogram. The sample was resuspended in 1.0 mL absolute ethanol and analyzed using the analytical HPLC method.

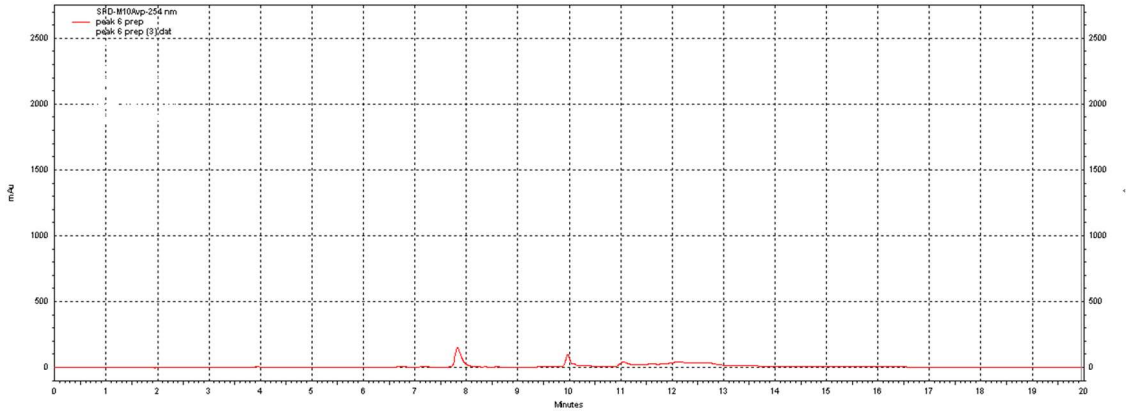

Figure 47: Chromatogram of the residue recovered representing peak 6 in the preparatory chromatogram. The sample was resuspended in 1.0 mL absolute ethanol and analyzed using the analytical HPLC method.

APPENDIX C

REPRESENTATIVE HPLC CHROMATOGRAMS FOR DECARBOXYLASE ACTIVITY IN VARIOUS CELL LINES

C1. Representative HPLC Chromoatograms for the Analysis of *Bacillus subtilis*

The following chromatograms were generated using an isocratic method of 30% HPLC-grade water (solvent A) and 70% HPLC-grade methanol (solvent B).

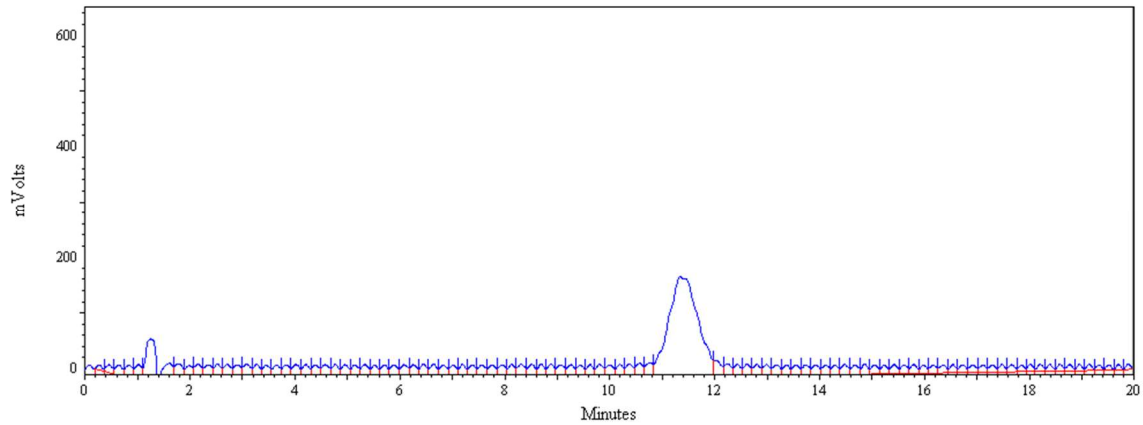

Figure 48: Chromatogram after 0 hours of incubation of CBDA with lysed *B. subtilis* induced with 40 mM salicylic acid.

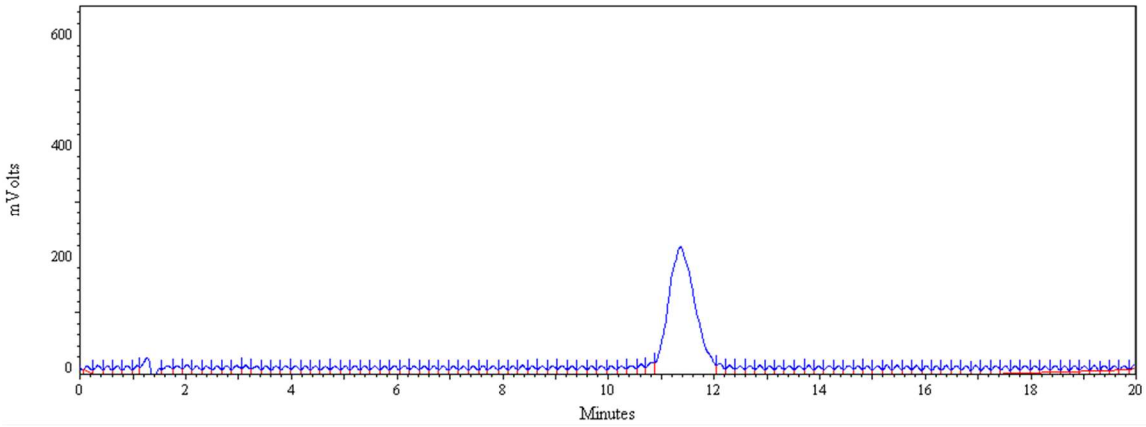

Figure 49: Chromatogram after 1 hour of incubation of CBDA with lysed *B. subtilis* induced with 40 mM salicylic acid.

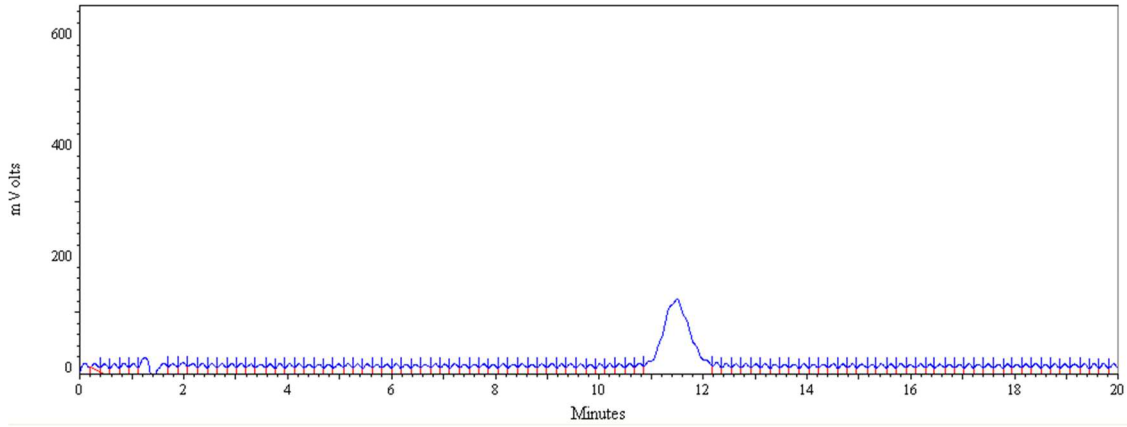

Figure 50: Chromatogram after 2 hours of incubation of CBDA with lysed *B. subtilis* induced with 40 mM salicylic acid.

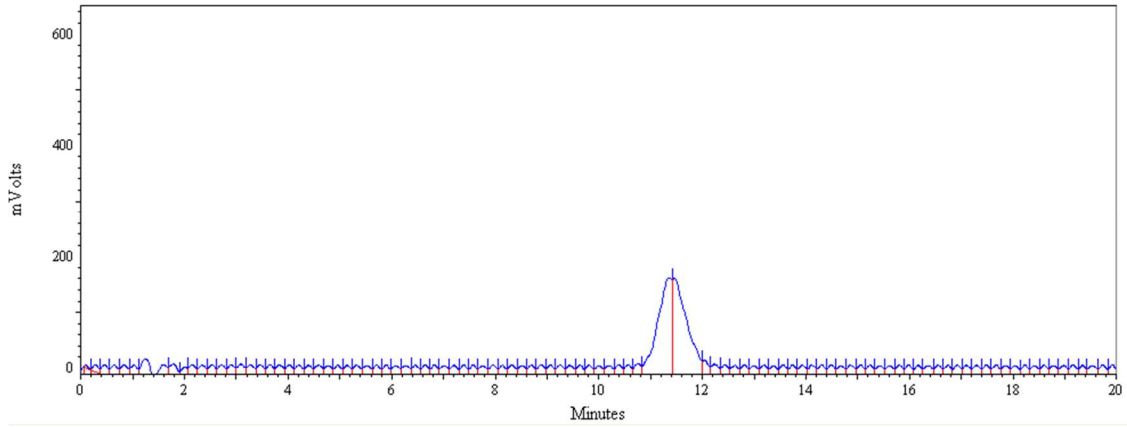

Figure 51: Chromatogram after 3 hours of incubation of CBDA with lysed *B. subtilis* induced with 40 mM salicylic acid.

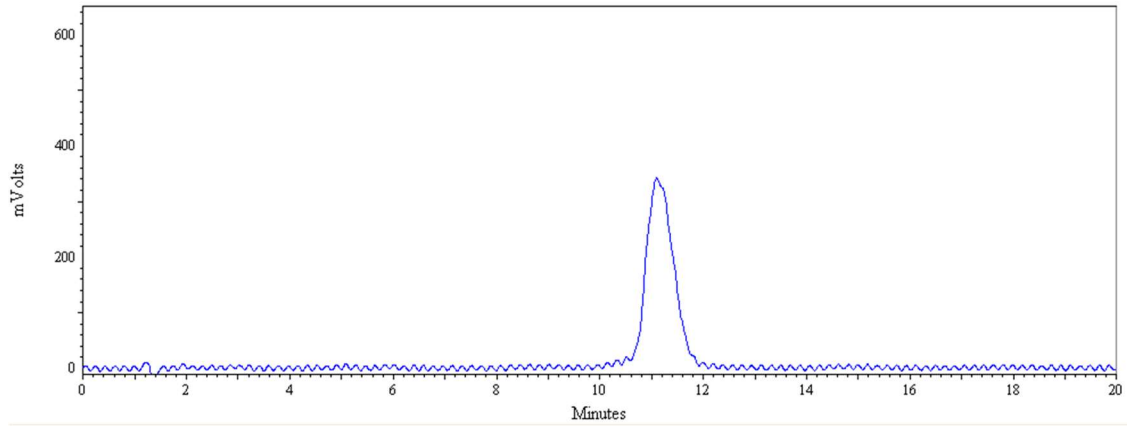

Figure 52: Chromatogram after 4 hours of incubation of CBDA with lysed *B. subtilis* induced with 40 mM salicylic acid.

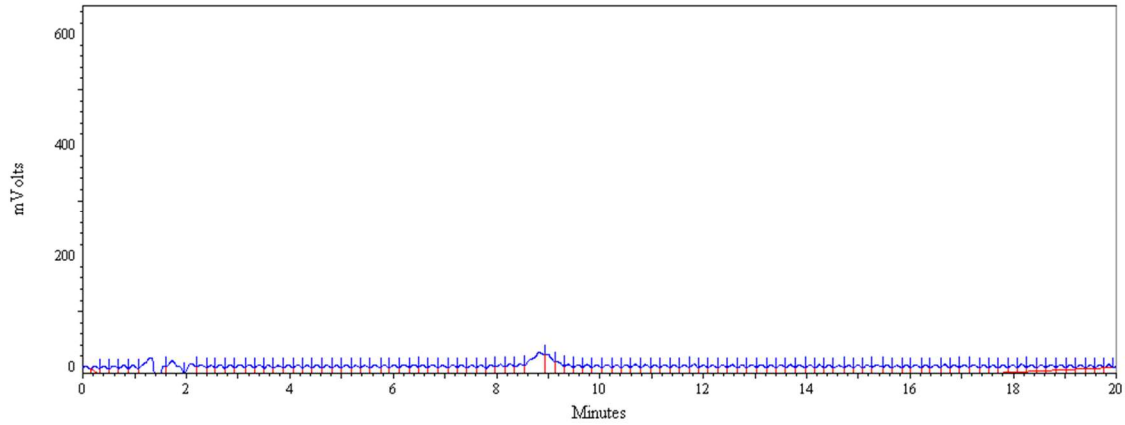

Figure 53: Chromatogram after 0 hours of incubation of CBDA with lysed *B. subtilis* induced with an unknown concentration of CBDA.

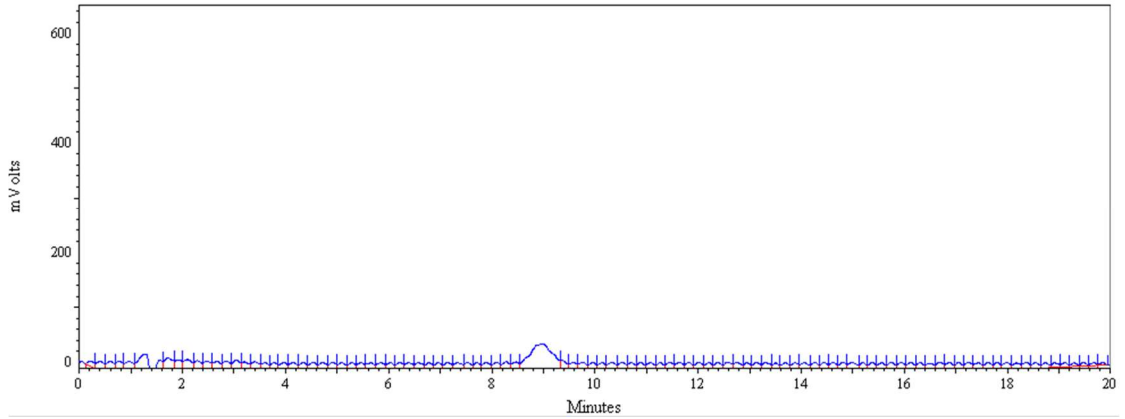

Figure 54: Chromatogram after 0.5 hour of incubation of CBDA with lysed *B. subtilis* induced with an unknown concentration of CBDA.

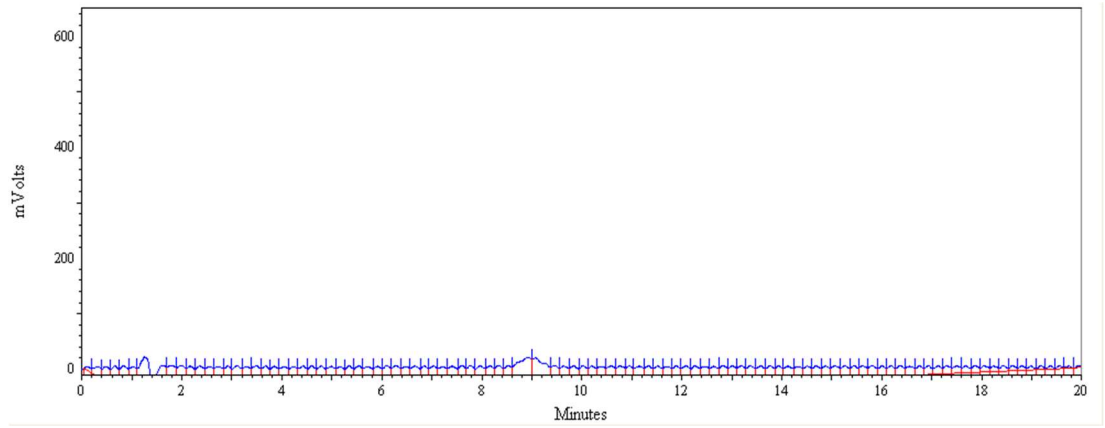

Figure 55: Chromatogram after 1 hour of incubation of CBDA with lysed *B. subtilis* induced with an unknown concentration of CBDA.

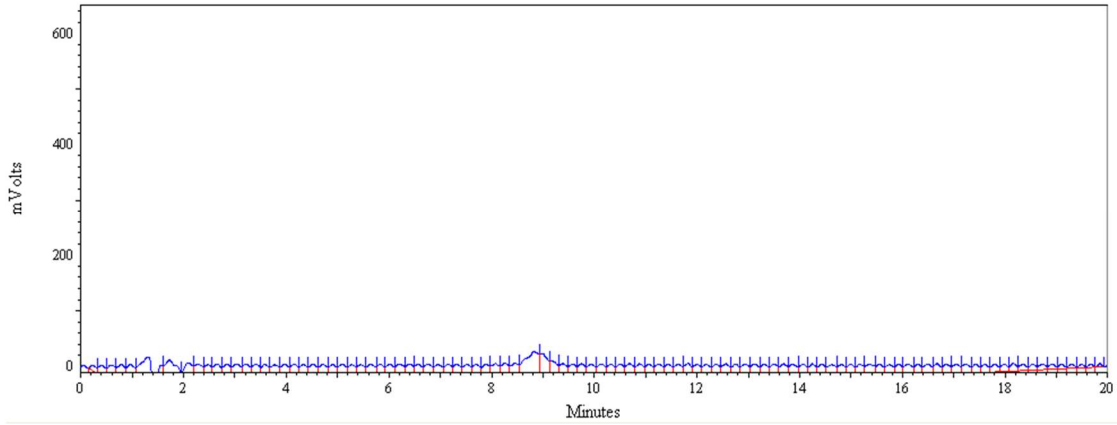

Figure 56: Chromatogram after 2 hours of incubation of CBDA with lysed *B. subtilis* induced with an unknown concentration of CBDA.

C2. Representative HPLC Chromoatograms for the Analysis of *Enterobacter (Klebsiella) aerogenes.*

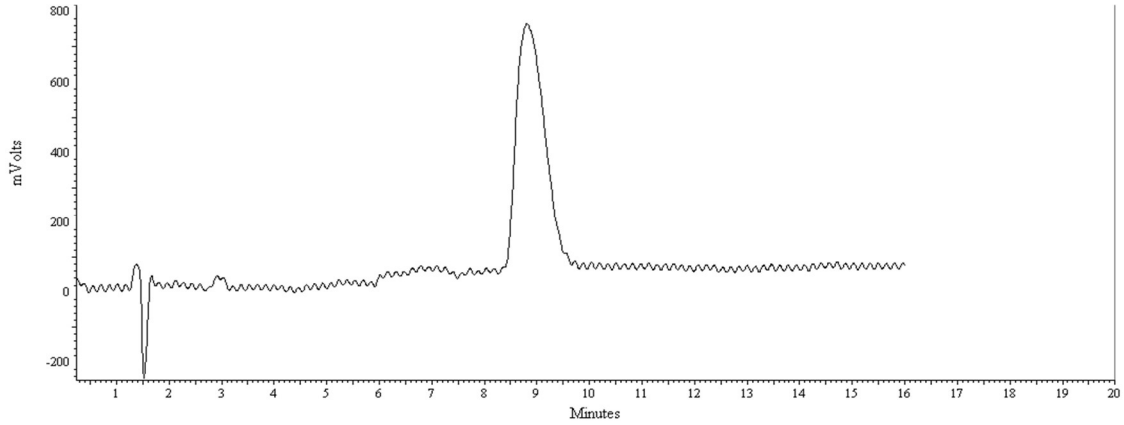

Figure 57: Chromatogram after 0 hours of incubation of CBDA with intact *E. aerogenes.*

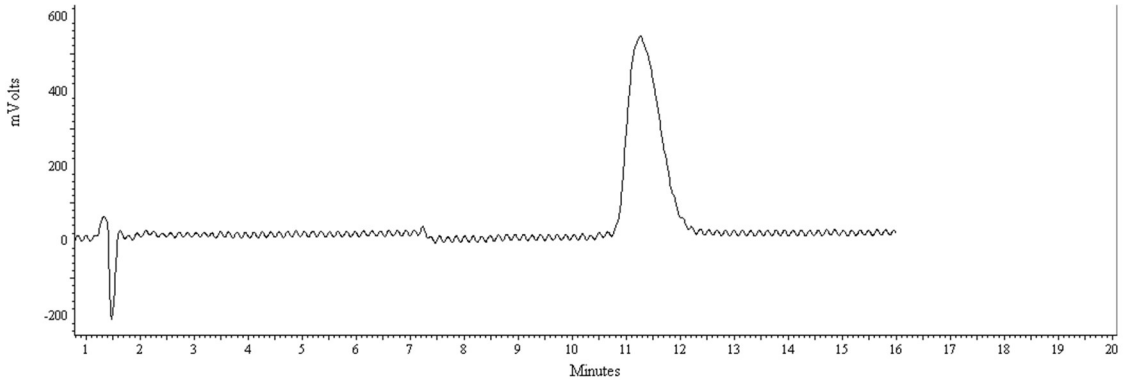

Figure 58: Chromatogram after 0.5 hour of incubation of CBDA with intact *E. aerogenes.*

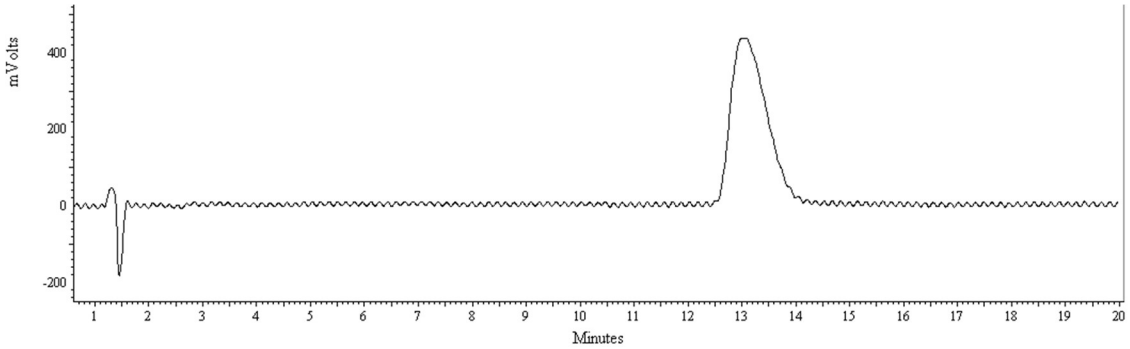

Figure 59: Chromatogram after 1 hour of incubation of CBDA with intact *E. aerogenes.*

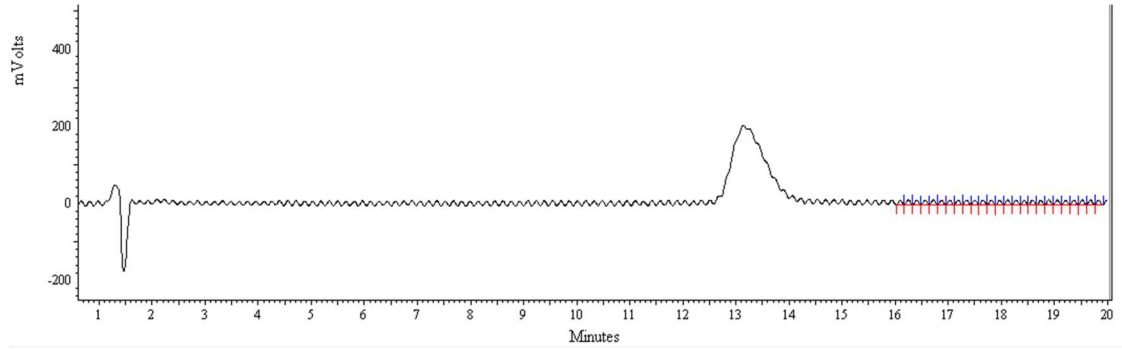

Figure 60: Chromatogram after 2 hours of incubation of CBDA with intact *E. aerogenes.*

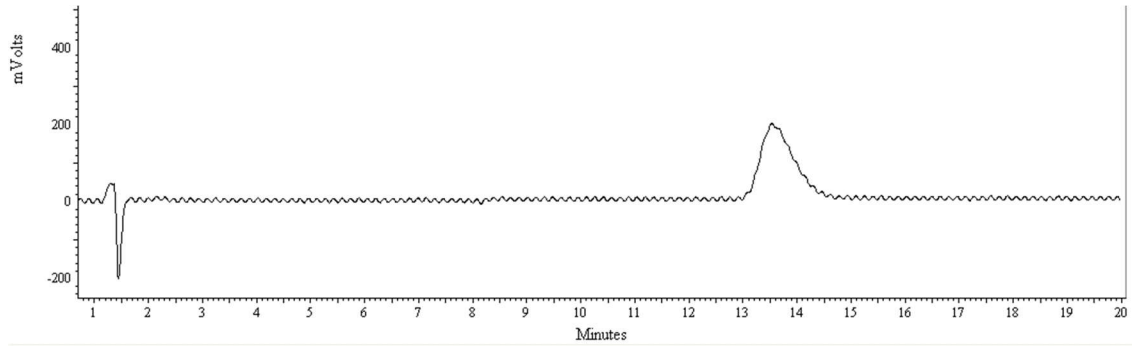

Figure 61: Chromatogram after 3 hours of incubation of CBDA with intact *E. aerogenes.*

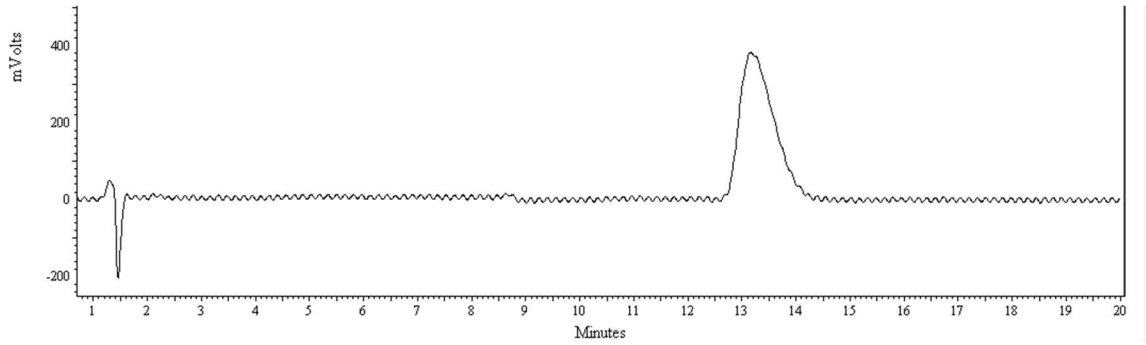

Figure 62: Chromatogram after 0 hours of incubation of CBDA with lysed *E. aerogenes.*

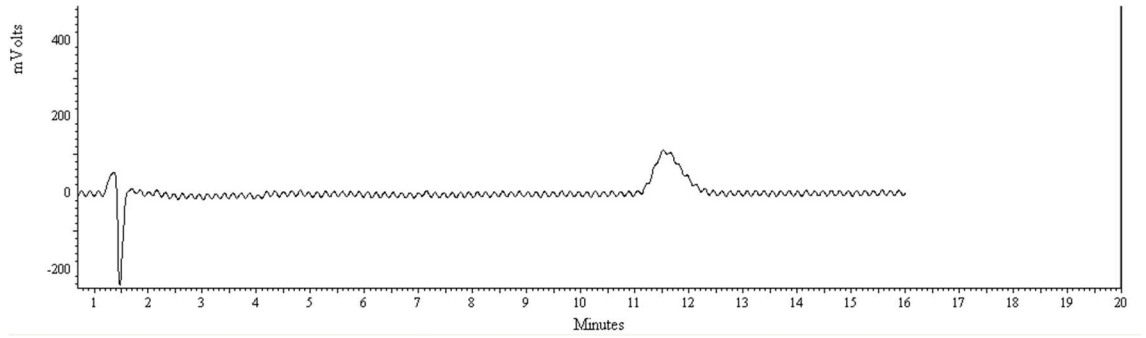

Figure 63: Chromatogram after 0.5 hour of incubation of CBDA with lysed *E. aerogenes.*

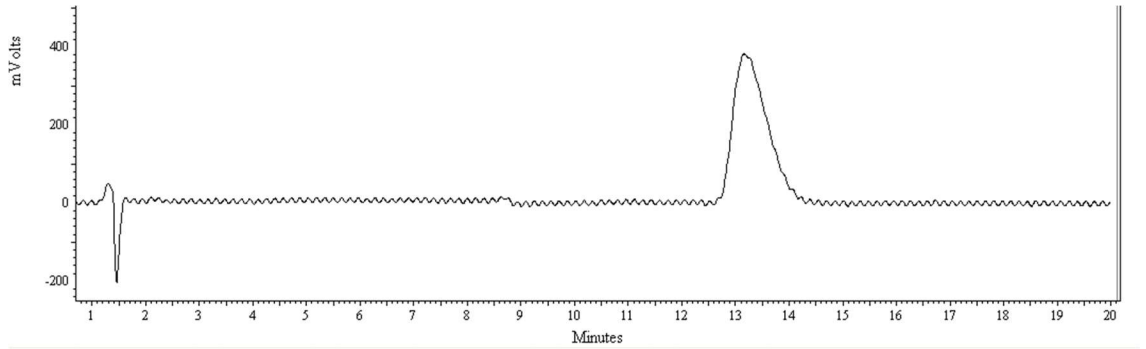

Figure 64: Chromatogram after 1 hour incubation of CBDA with lysed *E. aerogenes.*

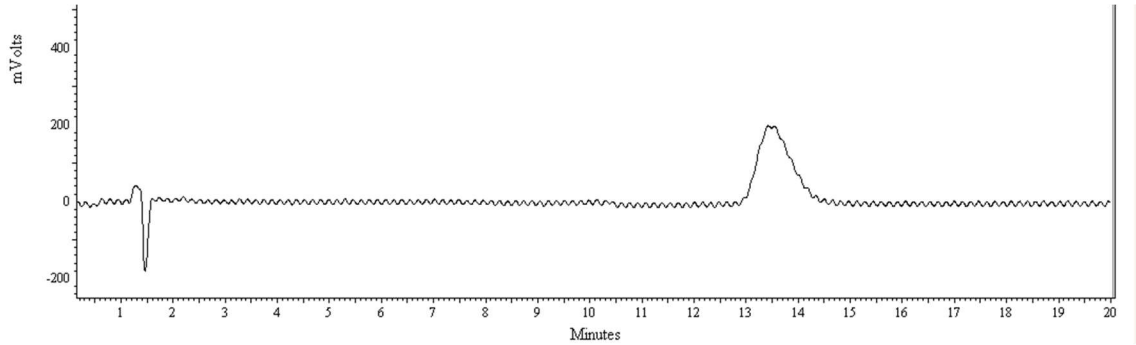

Figure 65: Chromatogram after 2 hours of incubation of CBDA with lysed *E. aerogenes*

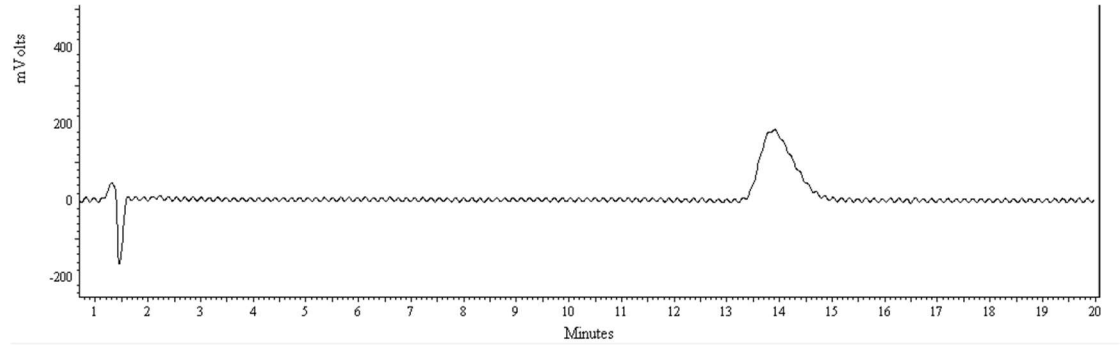

Figure 66: Chromatogram after 3 hours of incubation of CBDA with lysed *E. aerogenes*

C3. Representative HPLC Chromoatograms for the Analysis of *Klebsiella pneumonia*

The following chromatograms were generated using an isocratic method of 25% HPLC-grade water (solvent A) and 75% HPLC-grade methanol (solvent B).

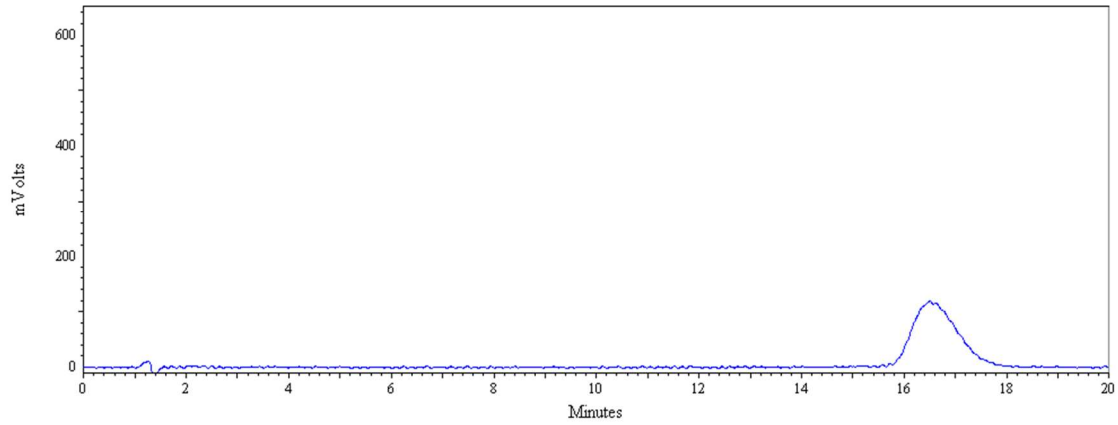

Figure 67: CBDA standard for *K. pneumonia* assay.

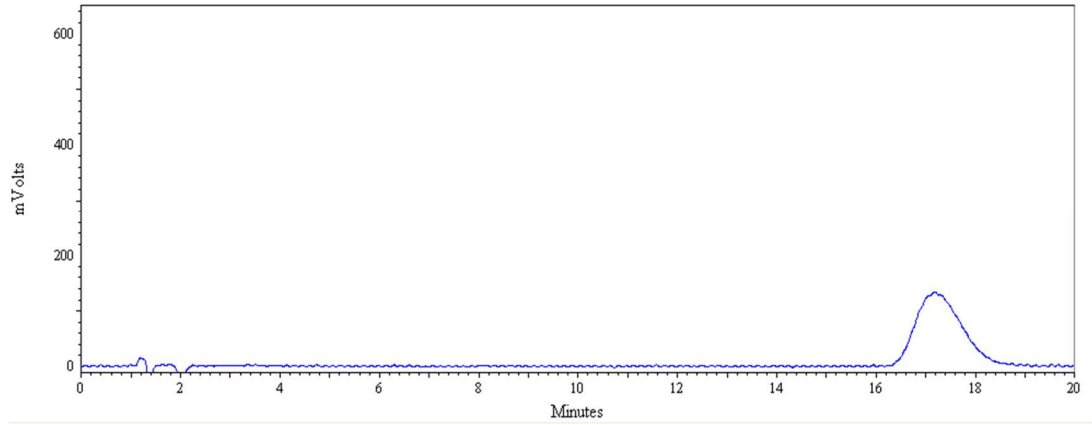

Figure 68: Chromatogram after 0 hour of incubation of CBDA with intact *K. pneumonia* induced with 30 mM 2,4-dihydroxybenzoic acid for 3 days.

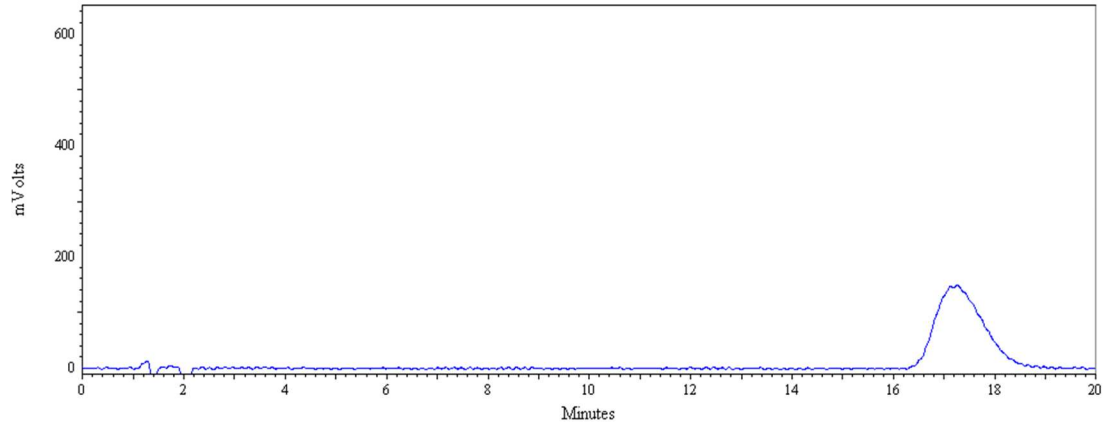

Figure 69: Chromatogram after 0.5 hour of incubation of CBDA with intact *K. pneumonia* induced with 30 mM 2,4-dihydroxybenzoic acid for 3 days.

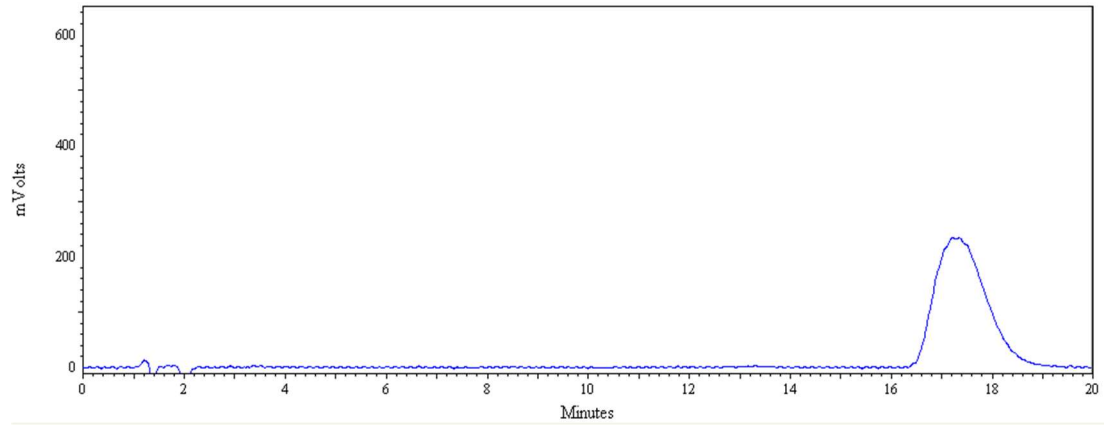

Figure 70: Chromatogram after 1 hour of incubation of CBDA with intact *K. pneumonia* induced with 30 mM 2,4-dihydroxybenzoic acid for 3 days.

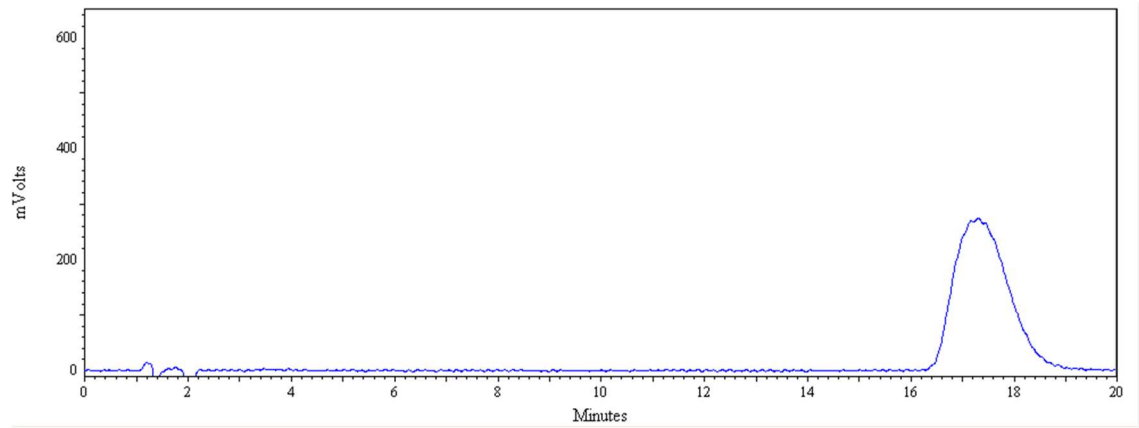

Figure 71: Chromatogram after 2 hours of incubation of CBDA with intact *K. pneumonia* induced with 30 mM 2,4-dihydroxybenzoic acid for 3 days.

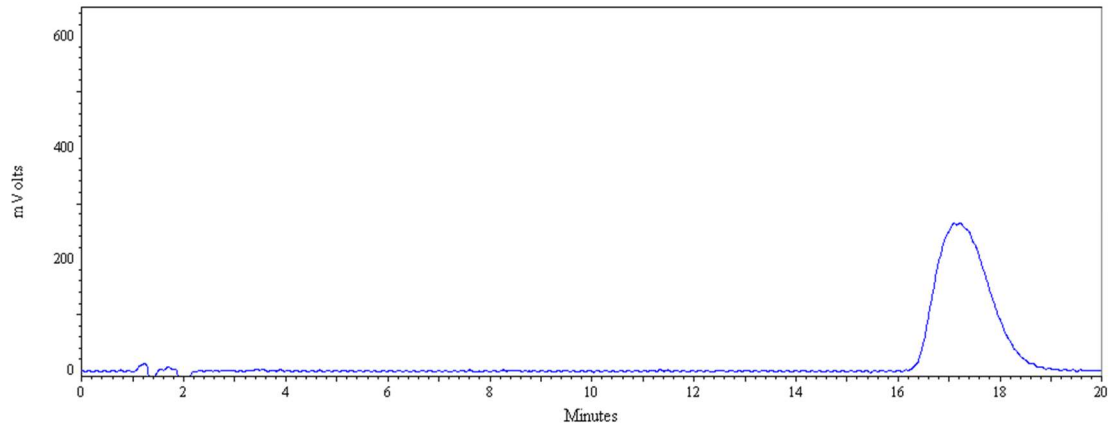

Figure 72: Chromatogram after 3 hours of incubation of CBDA with intact *K. pneumonia* induced with 30 mM 2,4-dihydroxybenzoic acid for 3 days.

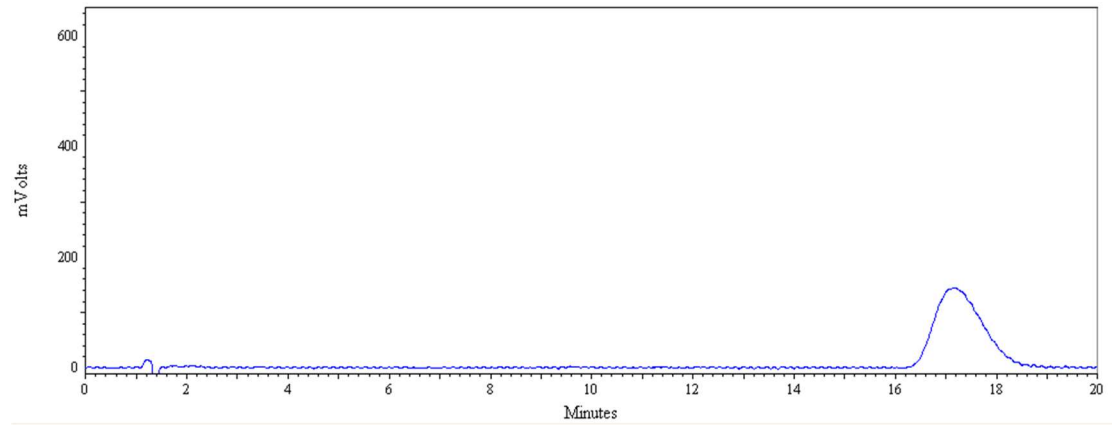

Figure 73: Chromatogram after 4 hours of incubation of CBDA with intact *K. pneumonia* induced with 30 mM 2,4-dihydroxybenzoic acid for 3 days.

C4. Representative HPLC Chromoatograms for the Analysis of *Aspergillus clavatus*

The following chromatograms were generated using an isocratic method of 25% HPLC-grade water (solvent A) and 75% HPLC-grade methanol (solvent B).

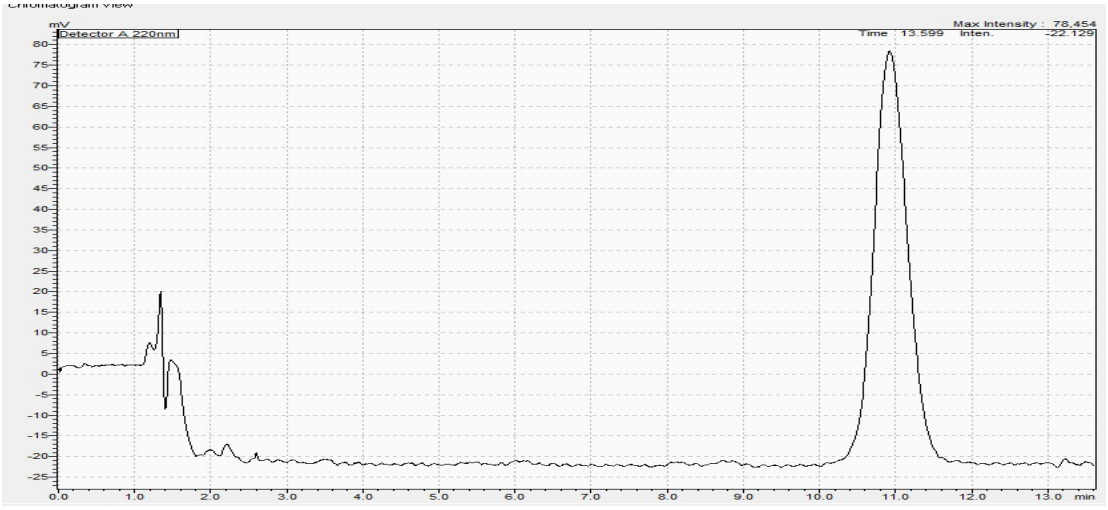

Figure 74: Chromatogram after 0 hour of incubation of CBDA with lysed *A. clavatus* Induced with 4 mM salicylic acid.

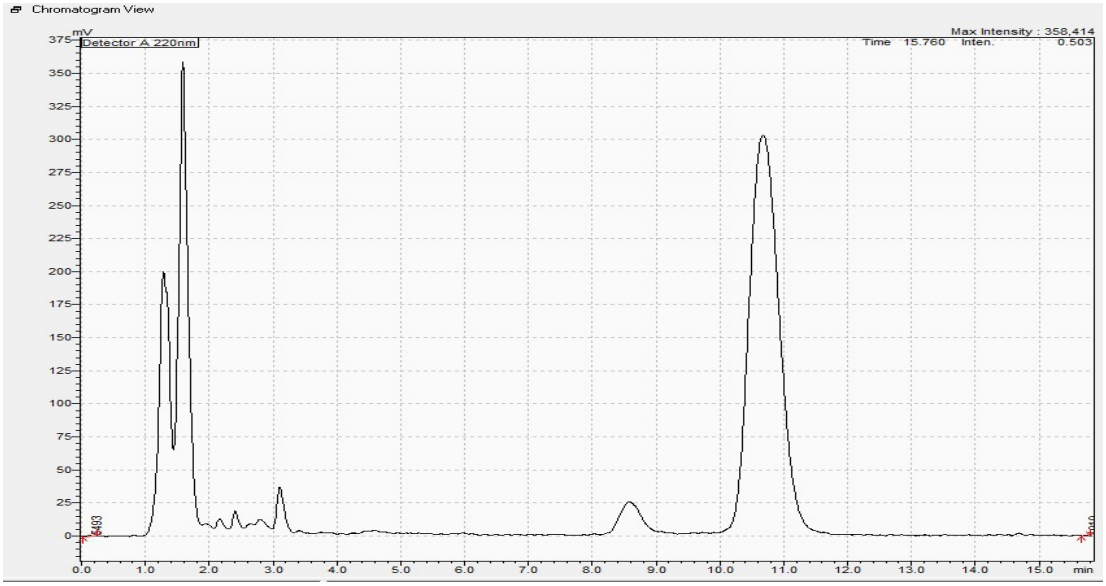

Figure 75: Chromatogram after 1 hour of incubation of CBDA with lysed *A. clavatus* induced with 4 mM salicylic acid.

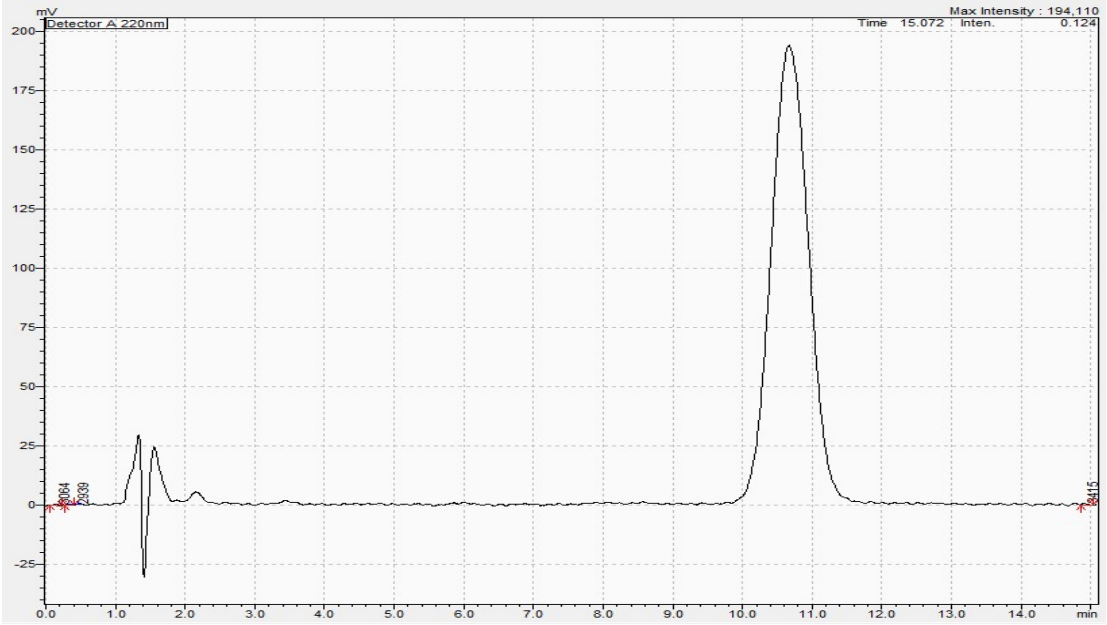

Figure 76: Chromatogram after 2 hours of incubation of CBDA with lysed *A. clavatus* induced with 4 mM salicylic acid.

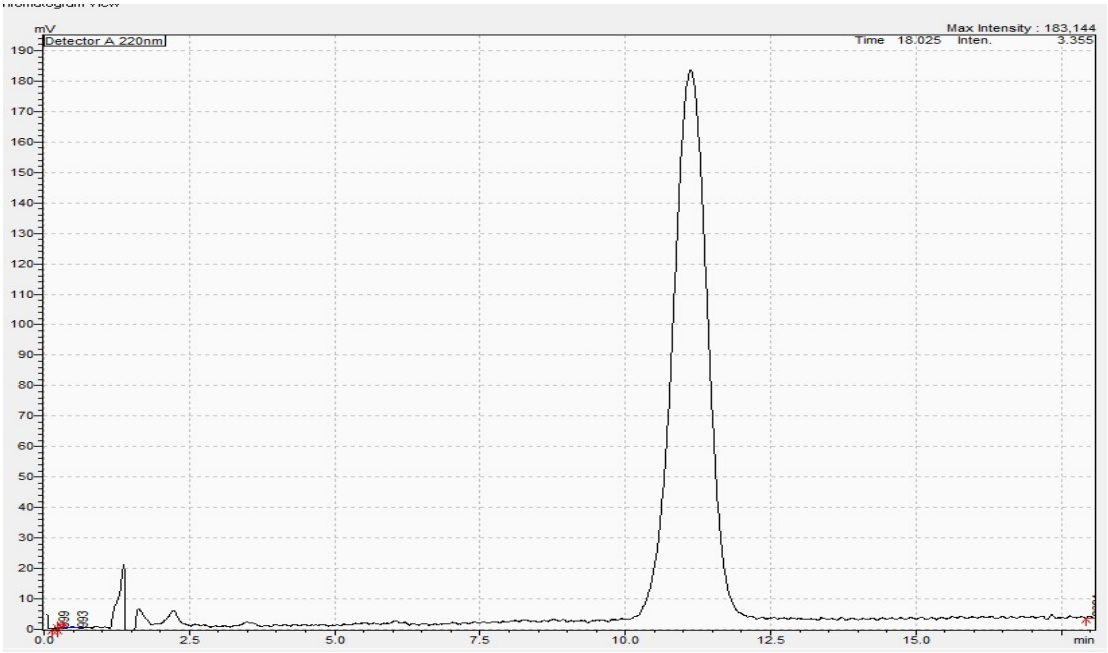

Figure 77: Chromatogram after 3 hours of incubation of CBDA with lysed *A. clavatus* induced with 4 mM salicylic acid.

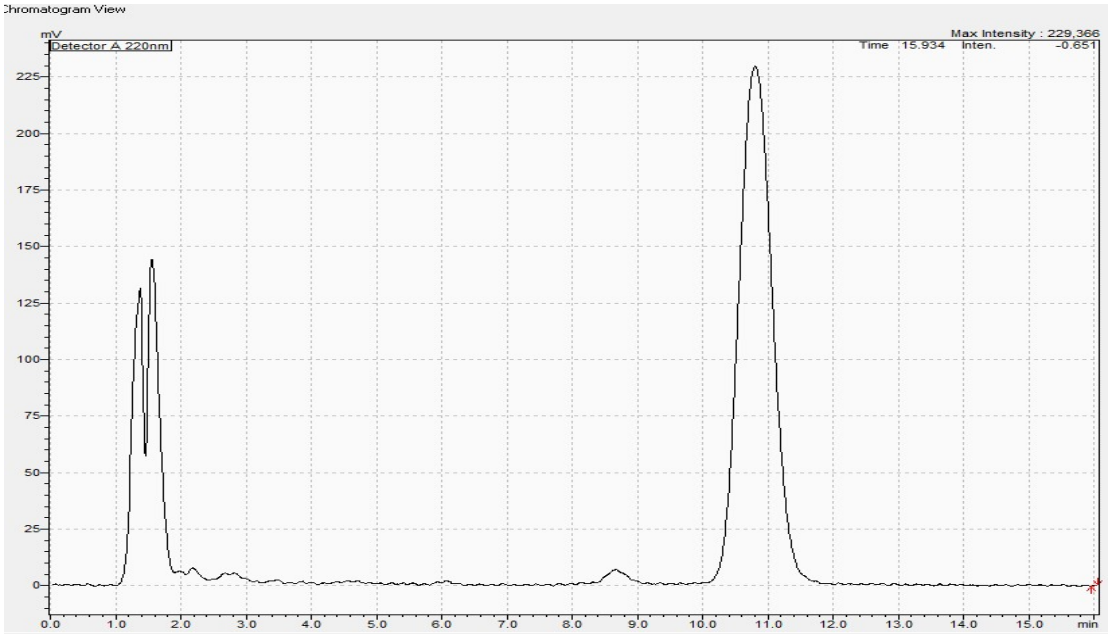

Figure 78: Chromatogram after 4 hours of incubation of CBDA with lysed *A. clavatus* Induced with 4 mM salicylic acid.

C5. Representative HPLC Chromoatograms for the Analysis of *Aspergillus niger*

The following chromatograms were generated using an isocratic method of 25% HPLC-grade water (solvent A) and 75% HPLC-grade methanol (solvent B).

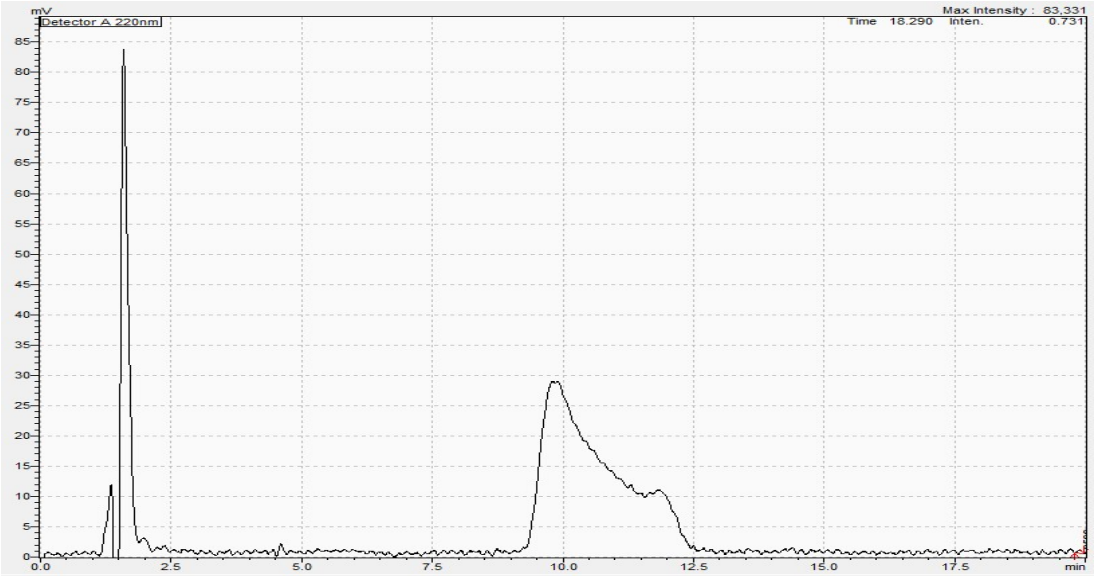

Figure 79: Chromatogram after 0 hour of incubation of CBDA with lysed *A. niger* induced with 4 mM salicylic acid.

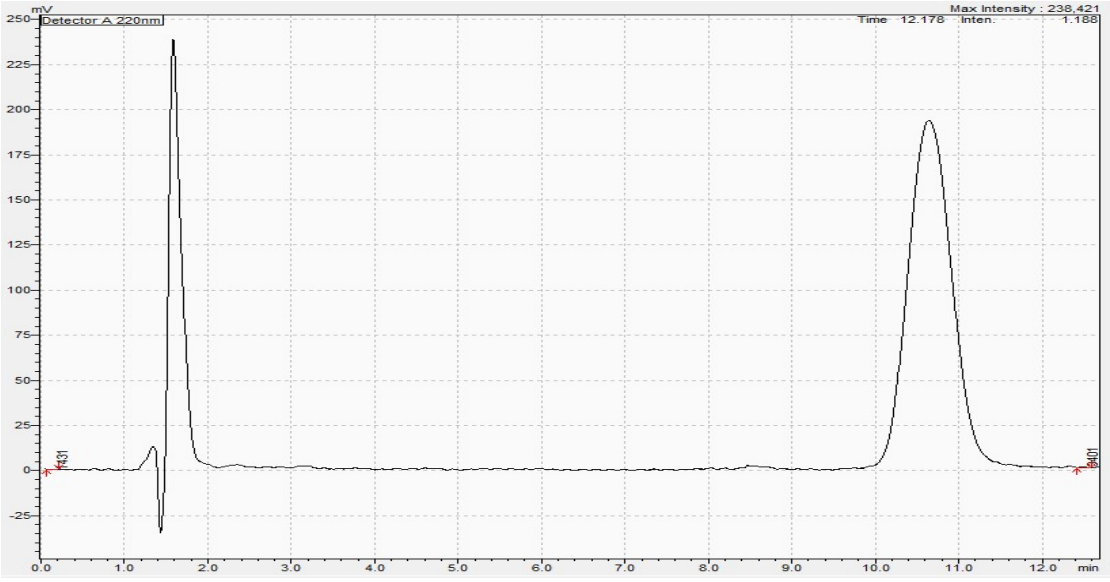

Figure 80: Chromatogram after 1 hour of incubation of CBDA with lysed *A. niger* induced with 4 mM salicylic acid.

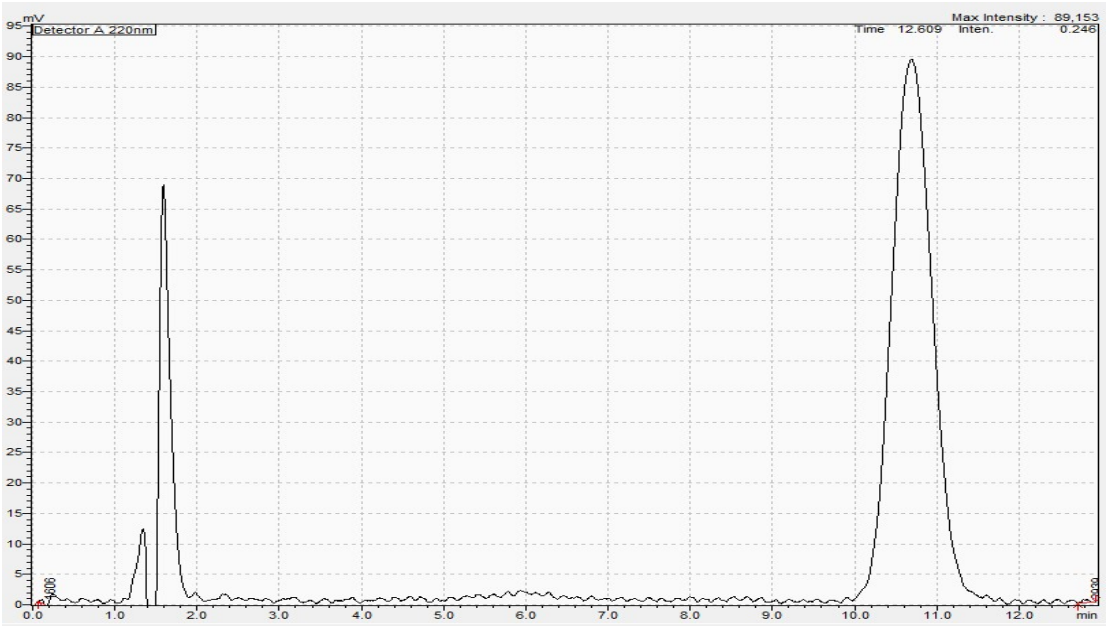

Figure 81: Chromatogram after 2 hours incubation of CBDA with lysed *A. niger* induced with 4 mM salicylic acid.

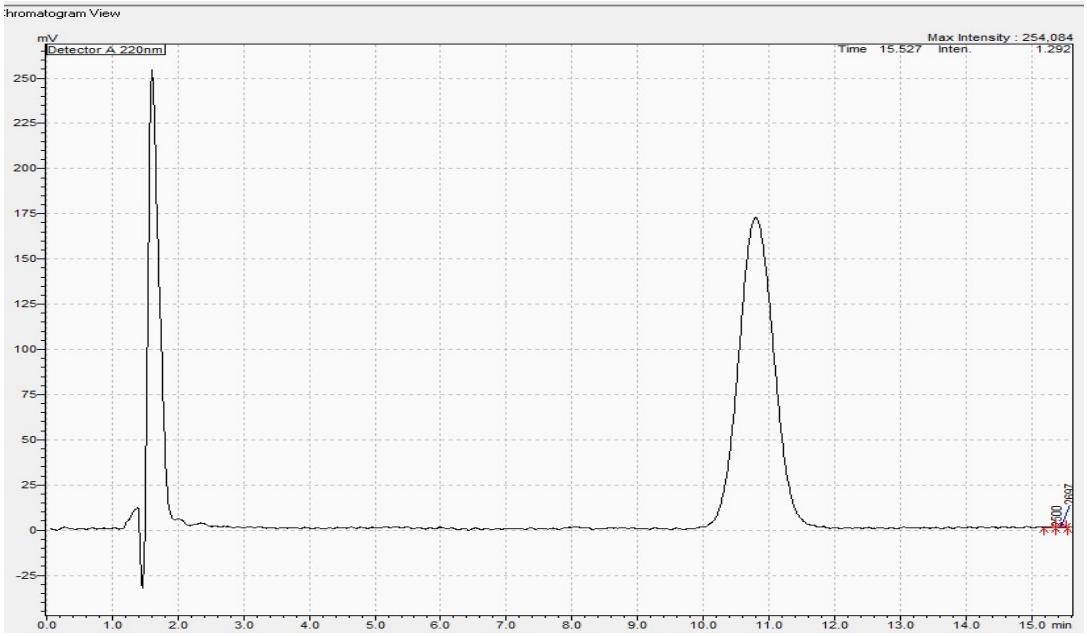

Figure 82: Chromatogram after 3 hours of incubation of CBDA with lysed *A. niger* induced with 4 mM salicylic acid.

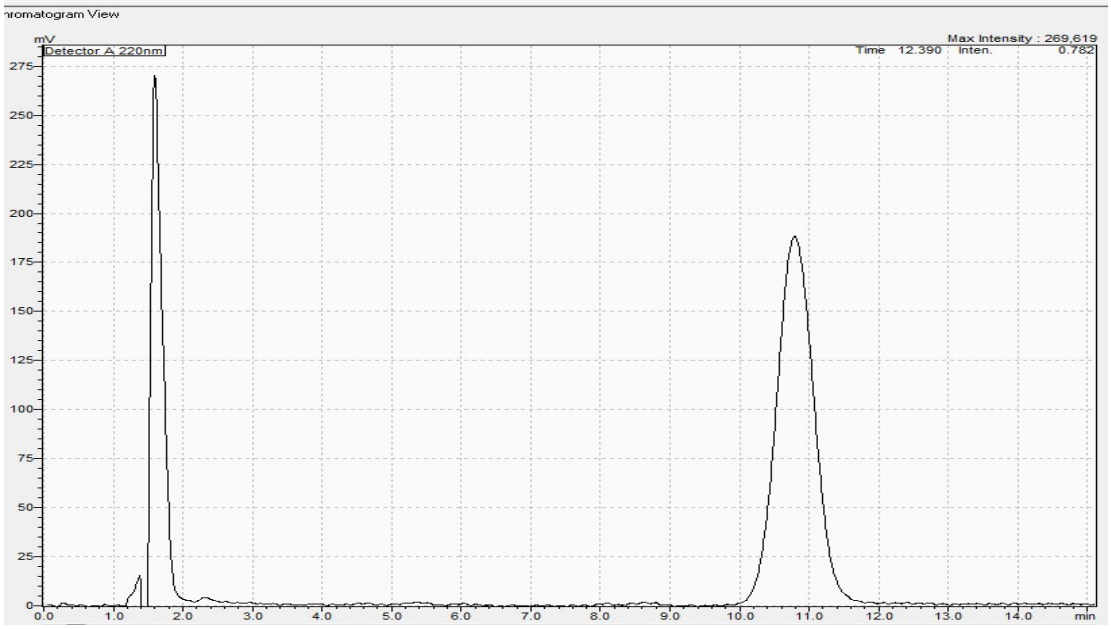

Figure 83: Chromatogram after 4 hours of incubation of CBDA with lysed *A. niger* induced with 4 mM salicylic acid.

C6. Representative HPLC Chromoatograms for the Analysis of *Penicillium chrysogenum*

The following chromatograms were generated using an isocratic method of 25% HPLC-grade water (solvent A) and 75% HPLC-grade methanol (solvent B).

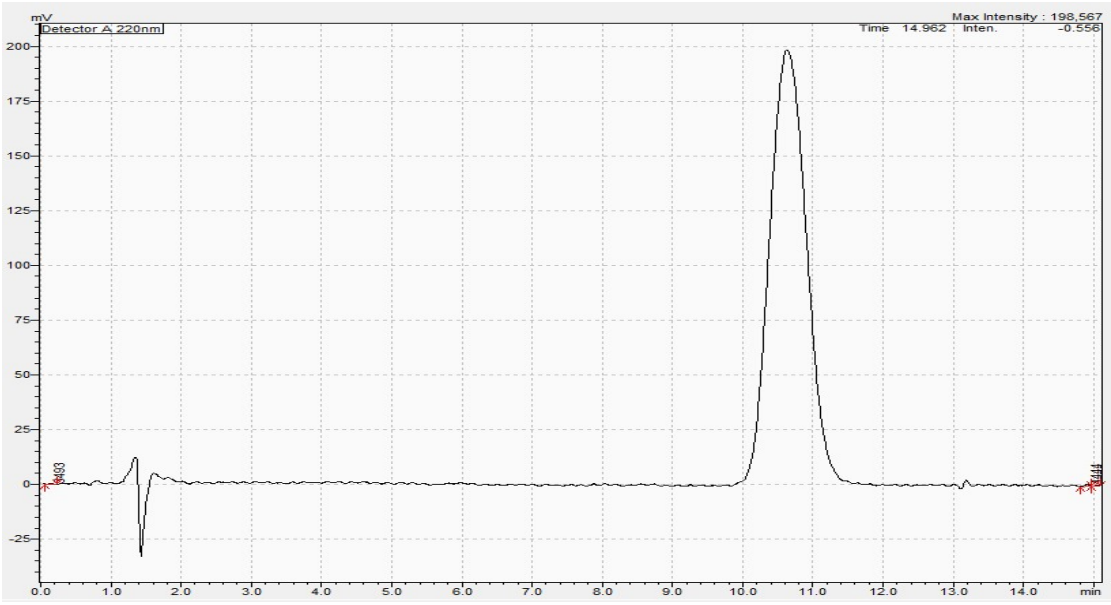

Figure 84: Chromatogram after 0 hours of incubation of CBDA with lysed *P. chrysogenum* induced with 4 mM salicylic acid.

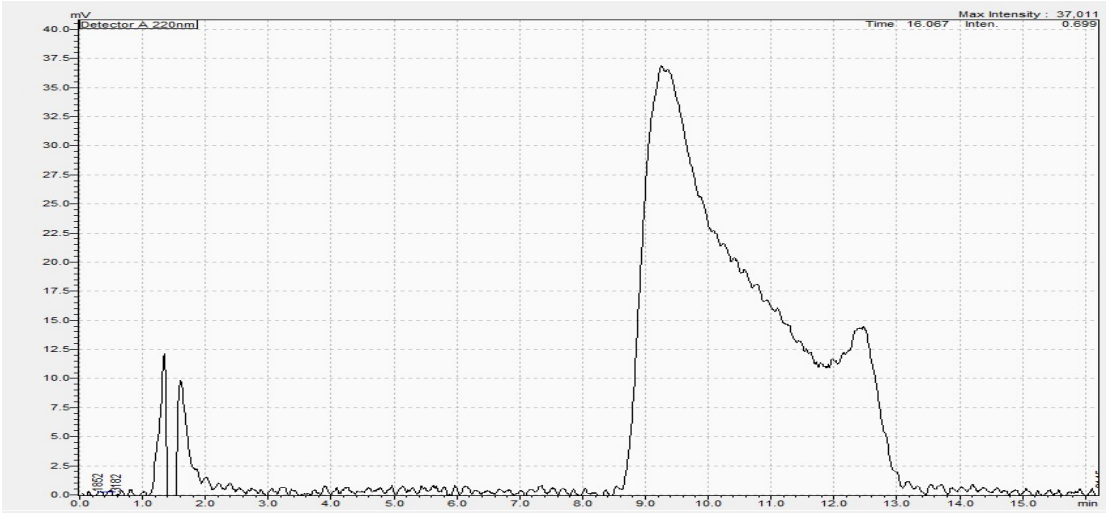

Figure 85: Chromatogram after 1 hour of incubation of CBDA with lysed *P. chrysogenum* induced with 4 mM salicylic acid.

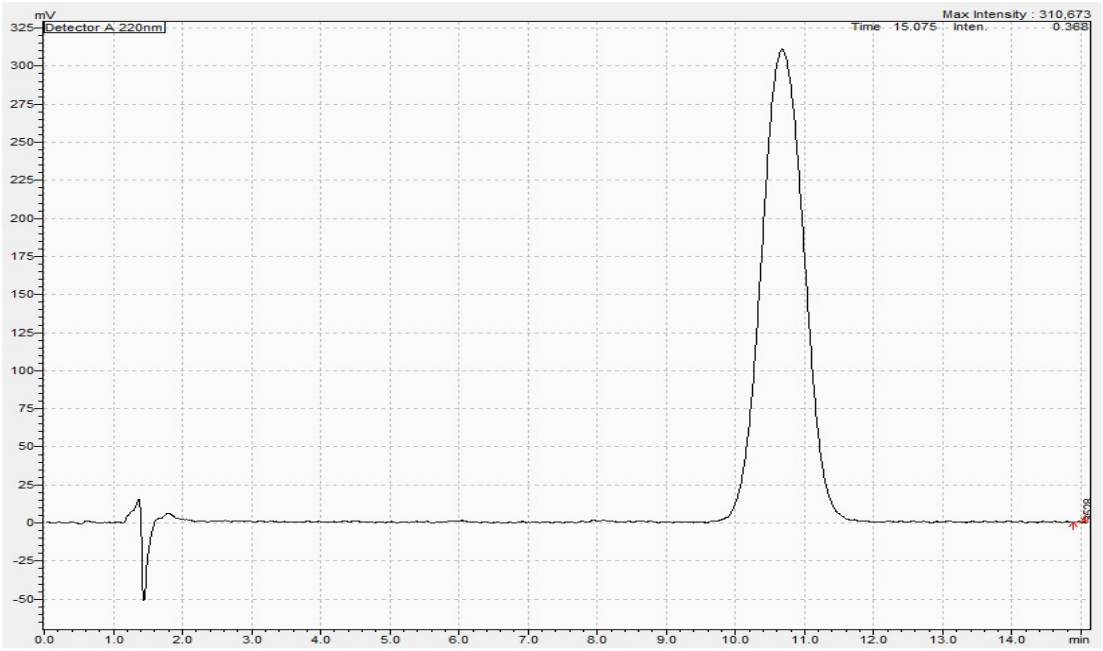

Figure 86: Chromatogram after 2 hours of incubation of CBDA with lysed *P. chrysogenum* induced with 4 mM salicylic acid.

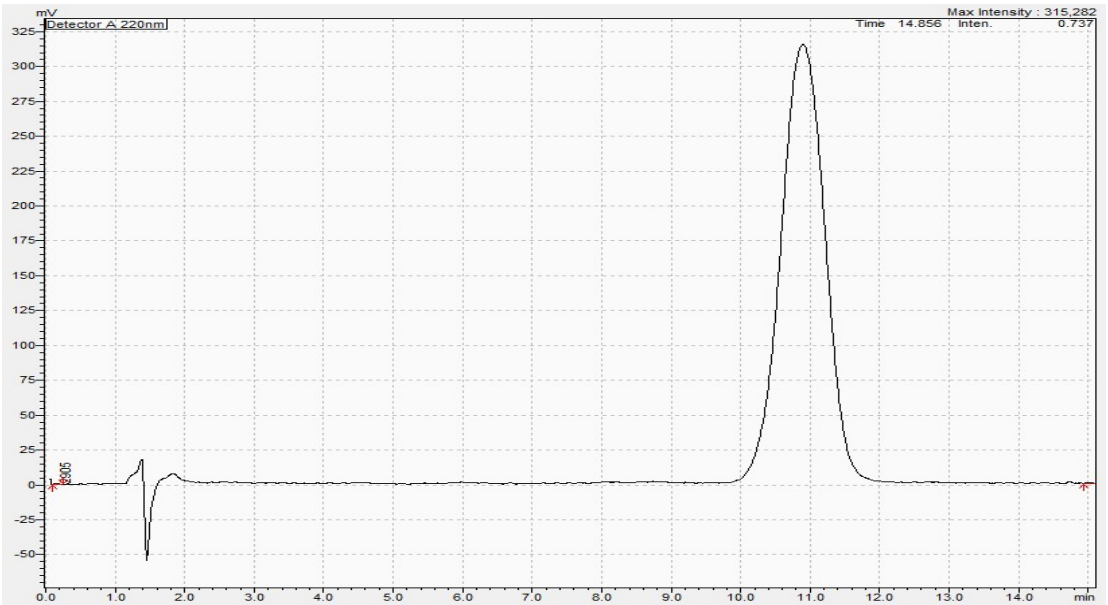

Figure 87: Chromatogram after 3 hours of incubation of CBDA with lysed *P. chrysogenum* induced with 4 mM salicylic acid.

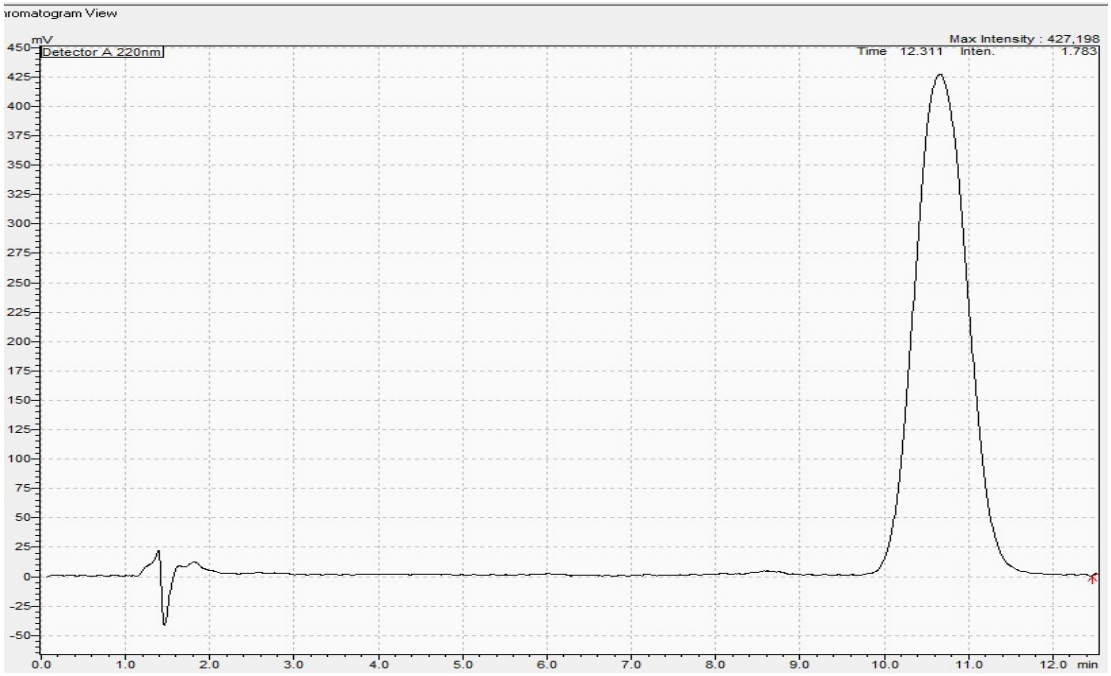

Figure 88: Chromatogram after 4 hours of incubation of CBDA with lysed *P. chrysogenum* induced with 4 mM salicylic acid.

C7. Representative HPLC Chromoatograms for the Analysis of *Pseudomonas putida*

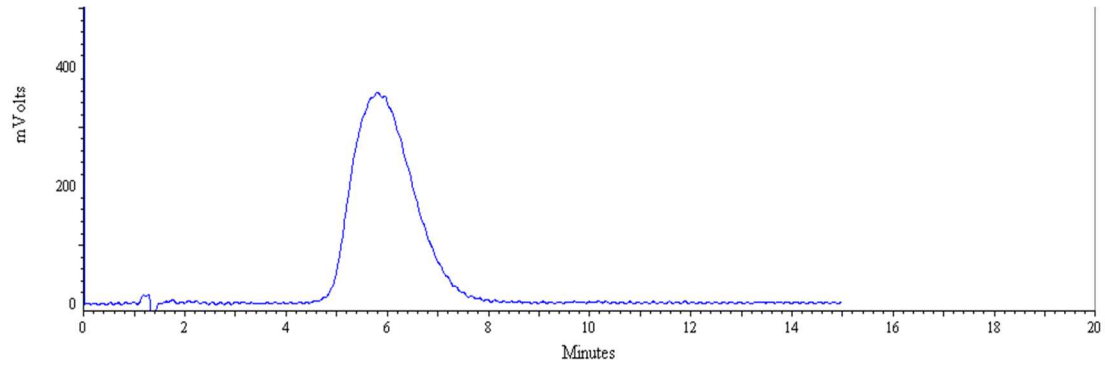

Figure 89: Chromatogram after 0 hour of incubation of CBDA with intact *P. putida.* Induced 1 day with 30 mM 2,4-dihydroxybenzoic acid.

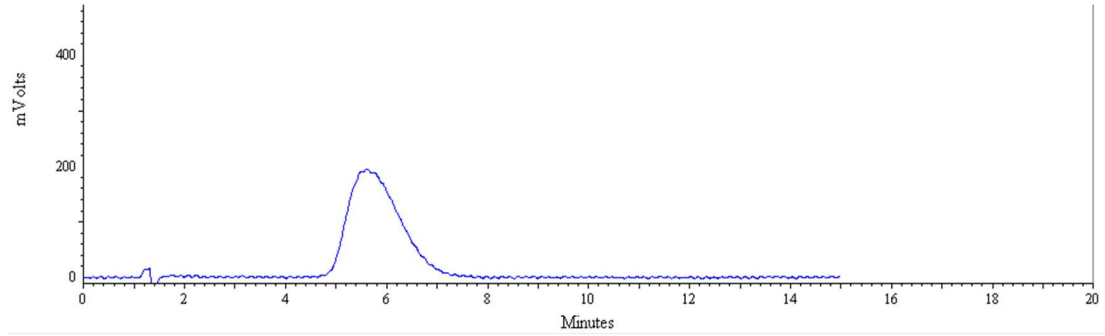

Figure 90: Chromatogram after 0.5 hour of incubation of CBDA with intact *P. putida.* Induced 1 day with 30 mM 2,4-dihydroxybenzoic acid.

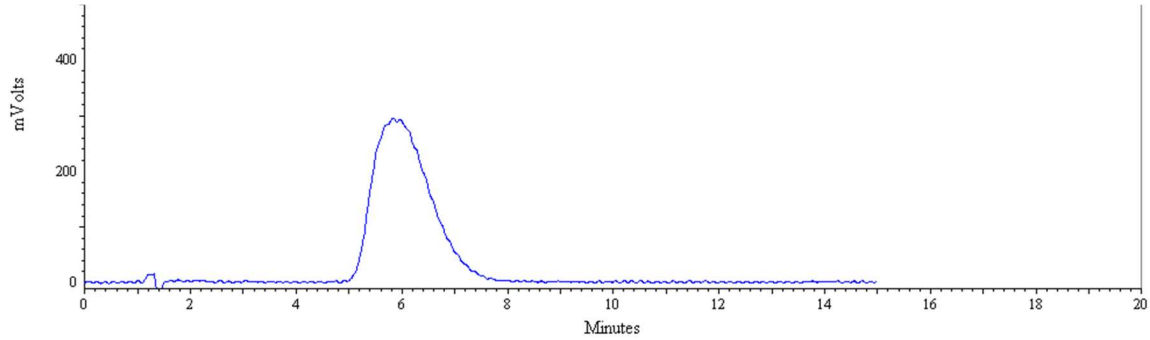

Figure 91: Chromatogram after 1 hour of incubation of CBDA with intact *P. putida.* Induced 1 day with 30 mM 2,4-dihydroxybenzoic acid.

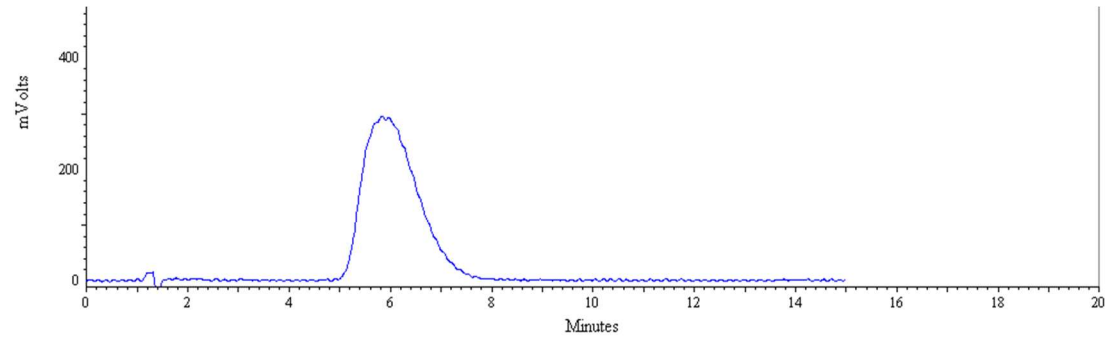

Figure 92: Chromatogram after 2 hours of incubation of CBDA with intact *P. putida.* Induced 1 day with 30 mM 2,4-dihydroxybenzoic acid.

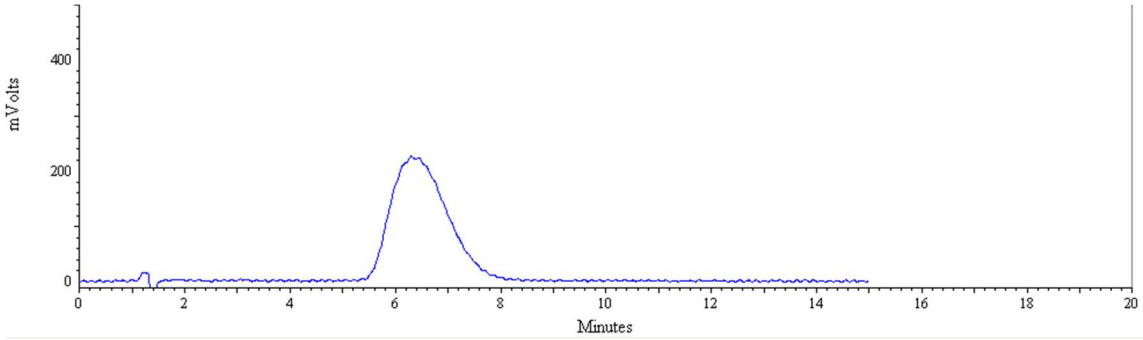

Figure 93: Chromatogram after 3 hours of incubation of CBDA with intact *P. putida.* Induced 1 day with 30 mM 2,4-dihydroxybenzoic acid.

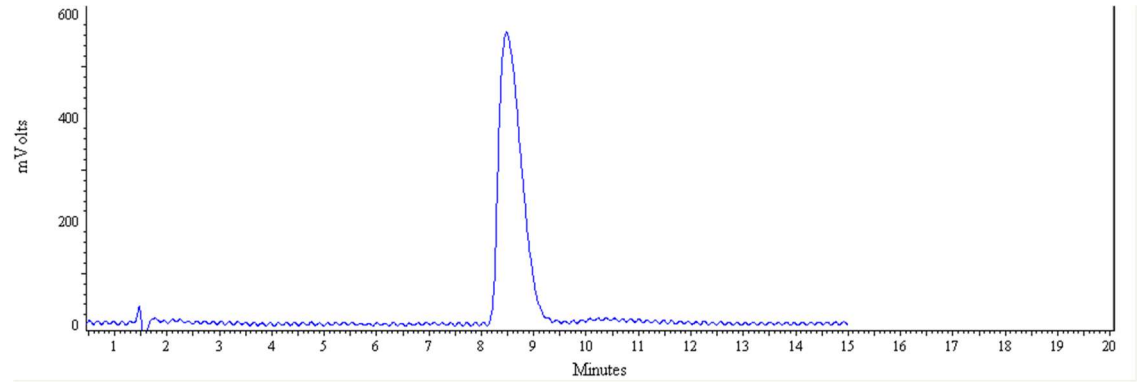

Figure 94: Chromatogram after 0 hour of incubation of CBDA with lysed *P. putida.* Induced 3 days with 30 mM 2,4-dihydroxybenzoic acid.

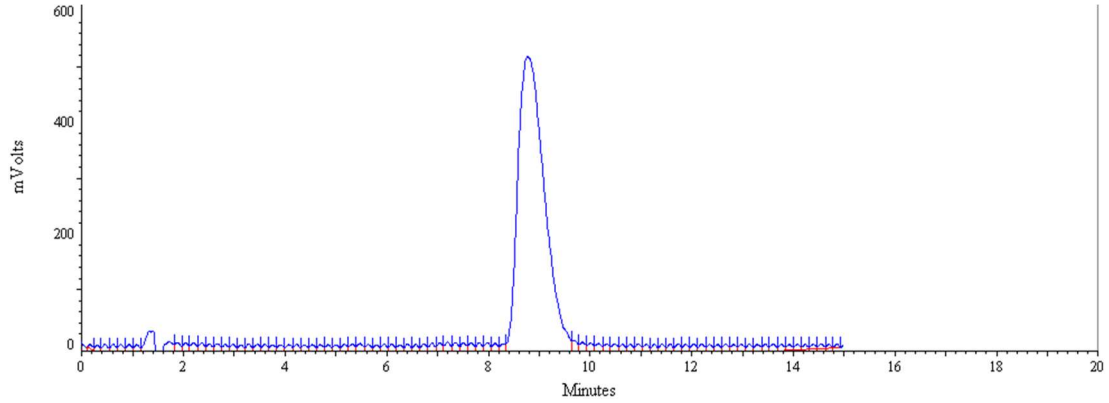

Figure 95: Chromatogram after 0.5 hour of incubation of CBDA with lysed *P. putida.* Induced 3 days with 30 mM 2,4-dihydroxybenzoic acid.

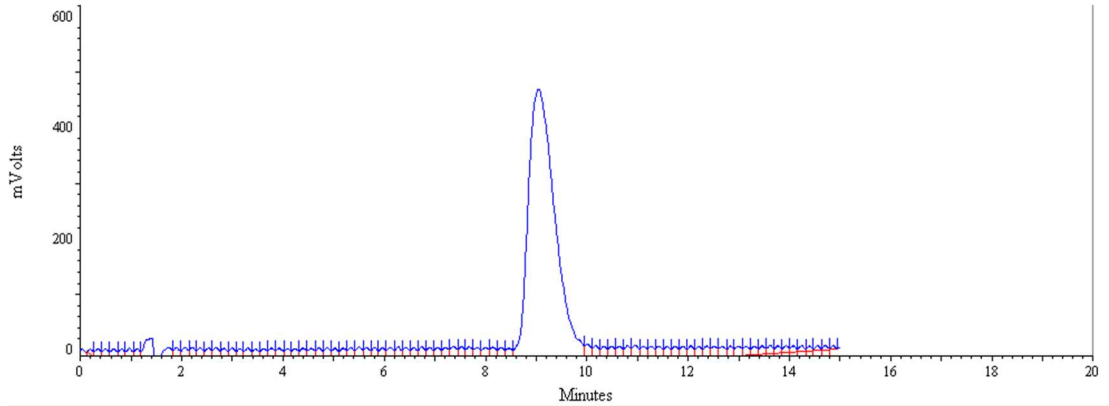

Figure 96: Chromatogram after 1 hour of incubation of CBDA with lysed *P. putida.* Induced 3 days with 30 mM 2,4-dihydroxybenzoic acid.

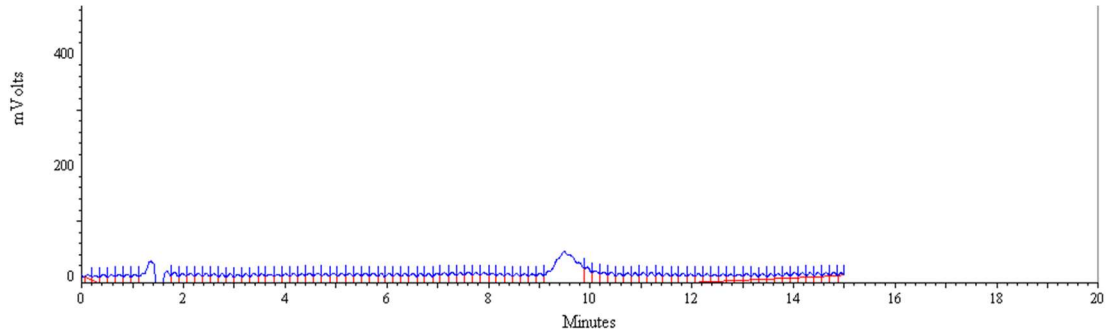

Figure 97: Chromatogram after 2 hours of incubation of CBDA with lysed *P. putida.* Induced 3 days with 30 mM 2,4-dihydroxybenzoic acid.

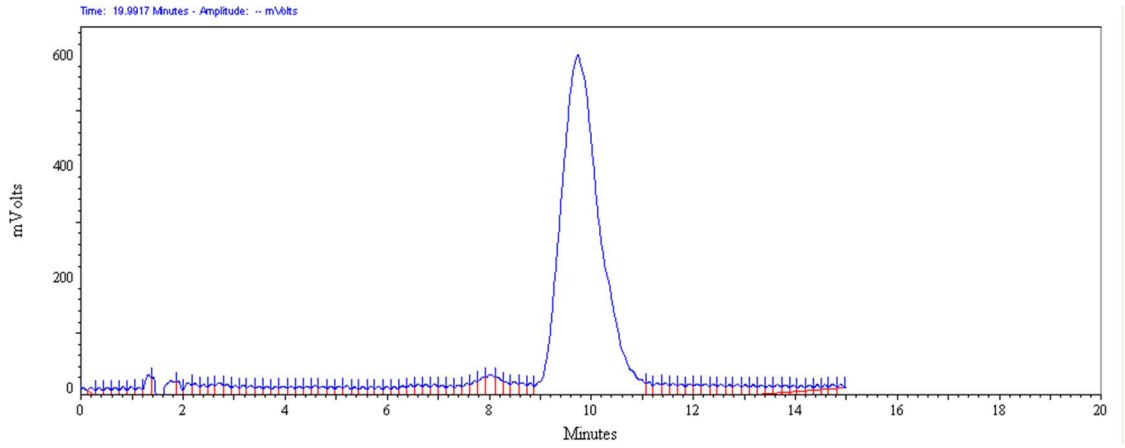

Figure 98: Chromatogram after 3 hours of incubation of CBDA with lysed *P. putida* induced 3 days with 30 mM 2,4-dihydroxybenzoic acid.

The following chromatograms were generated according to the analytical procedure outlined in Instrumentation of the Methodology section.

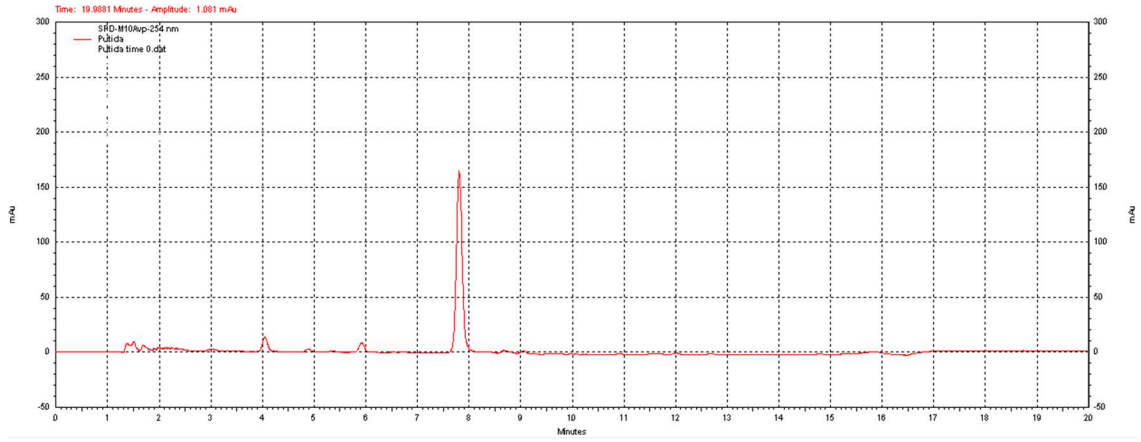

Figure 99: Chromatogram after 0 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

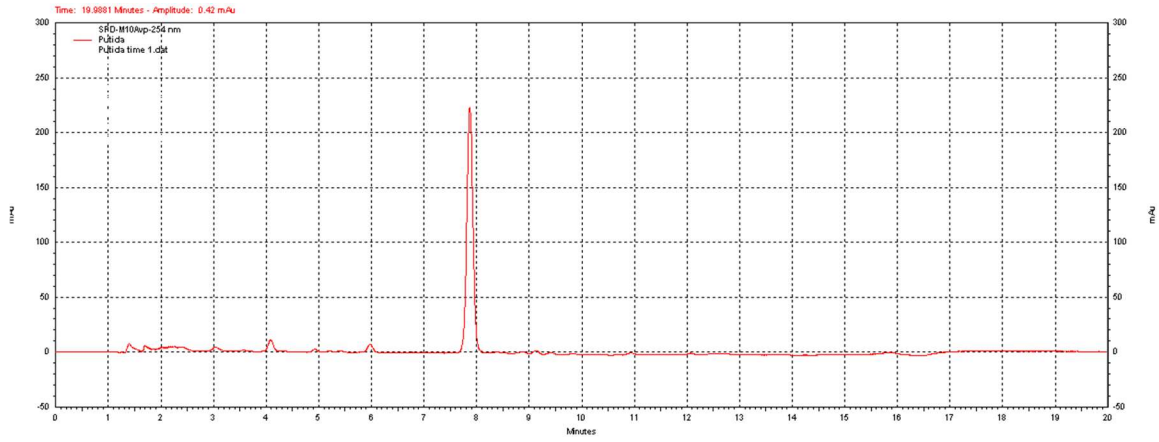

Figure 100: Chromatogram after 1 hour of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

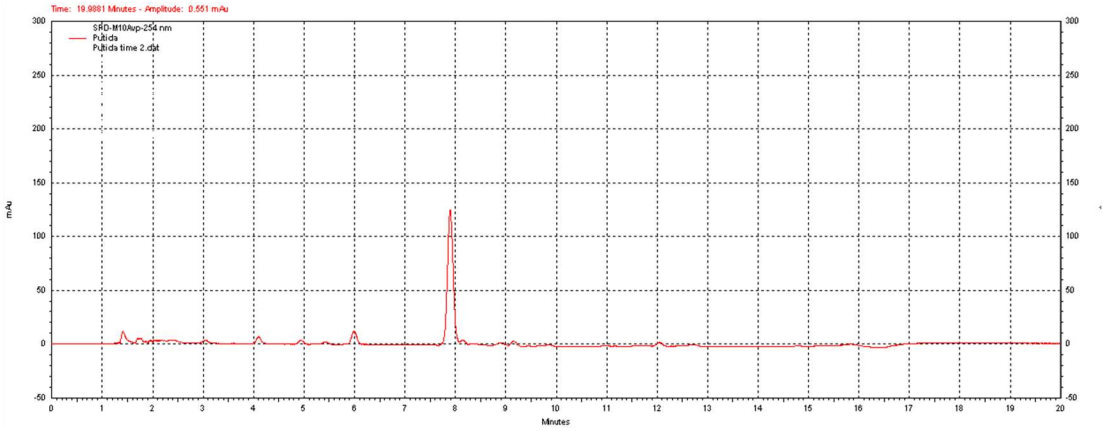

Figure 101: Chromatogram after 2 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

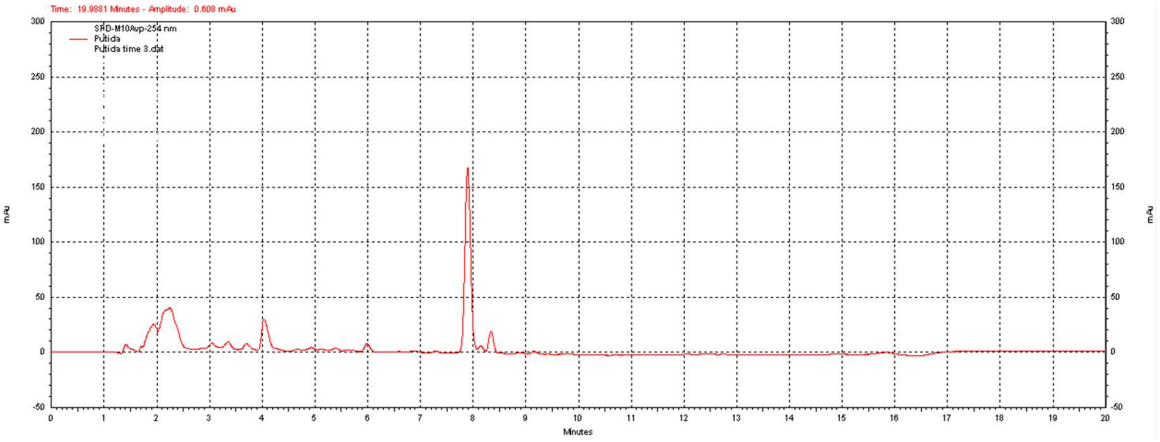

Figure 102: Chromatogram after 3 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

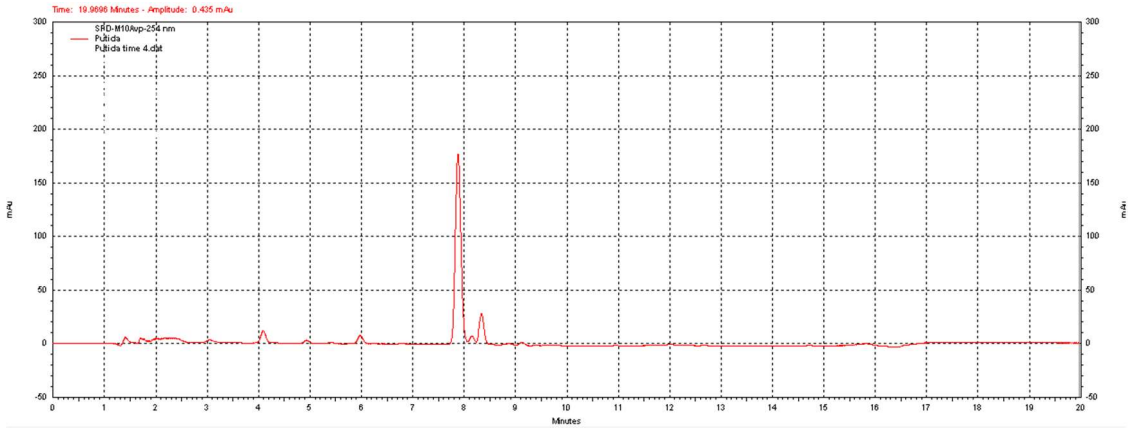

Figure 103: Chromatogram after 4 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

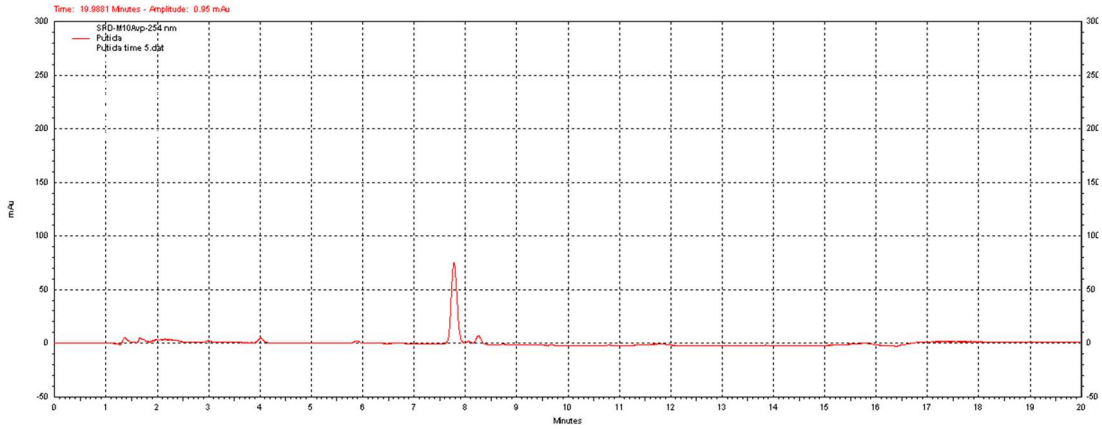

Figure 104: Chromatogram after 5 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

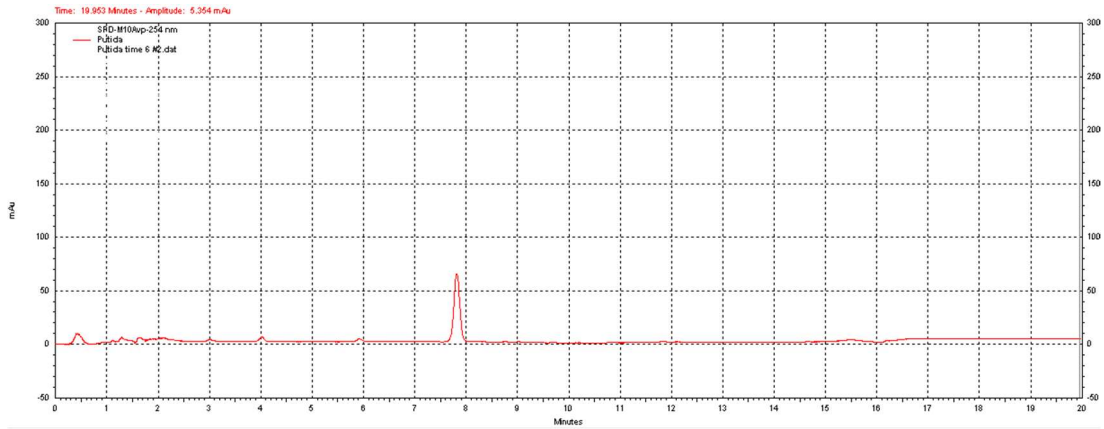

Figure 105: Chromatogram after 6 hours of incubation of CBDA with lysed *P. putida* I nduced with 10 mM salicylic acid.

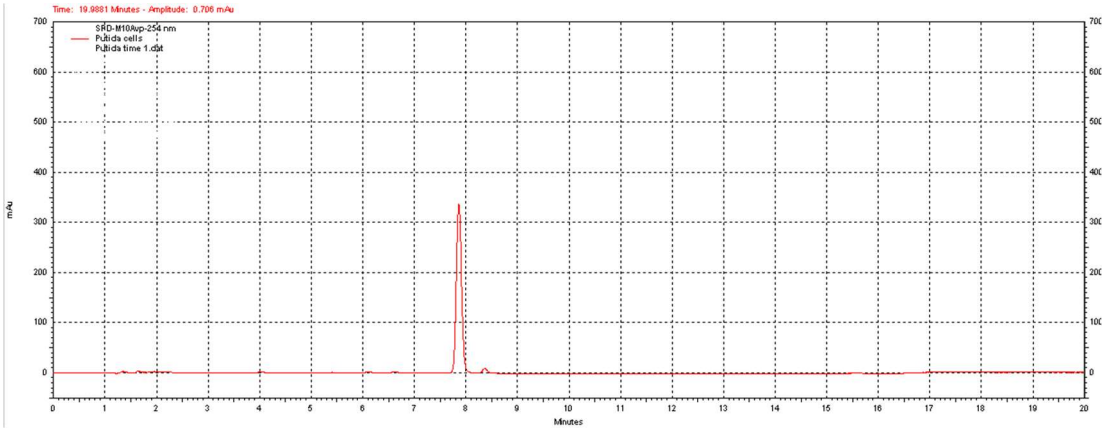

Figure 106: Chromatogram after 0 hour of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

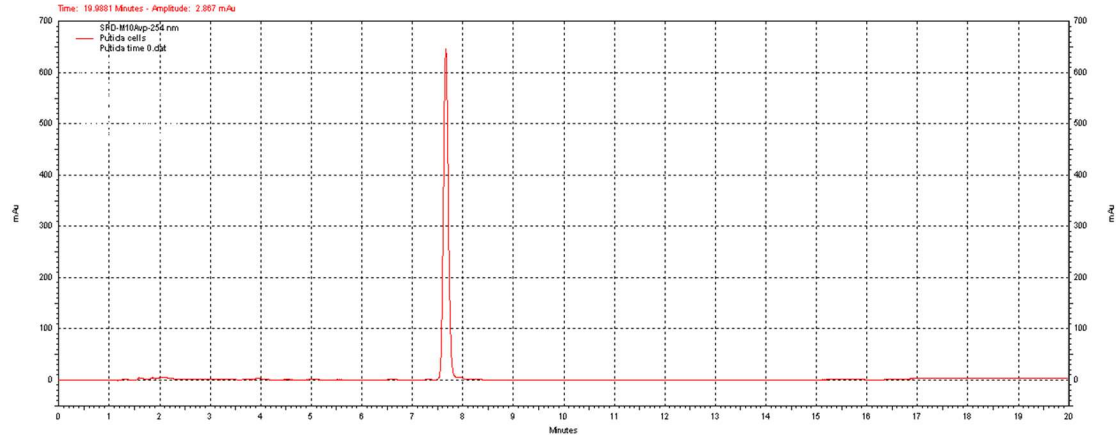

Figure 107: Chromatogram after 1 hour of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

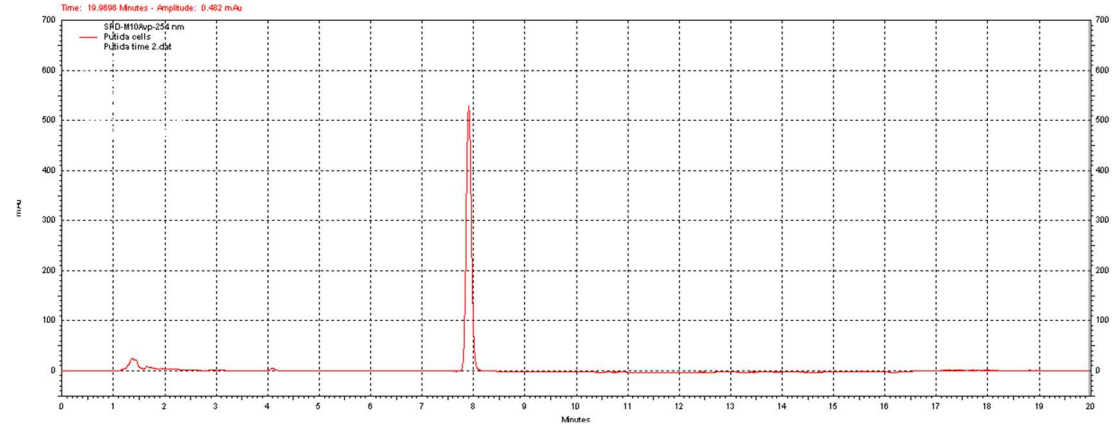

Figure 108: Chromatogram after 2 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

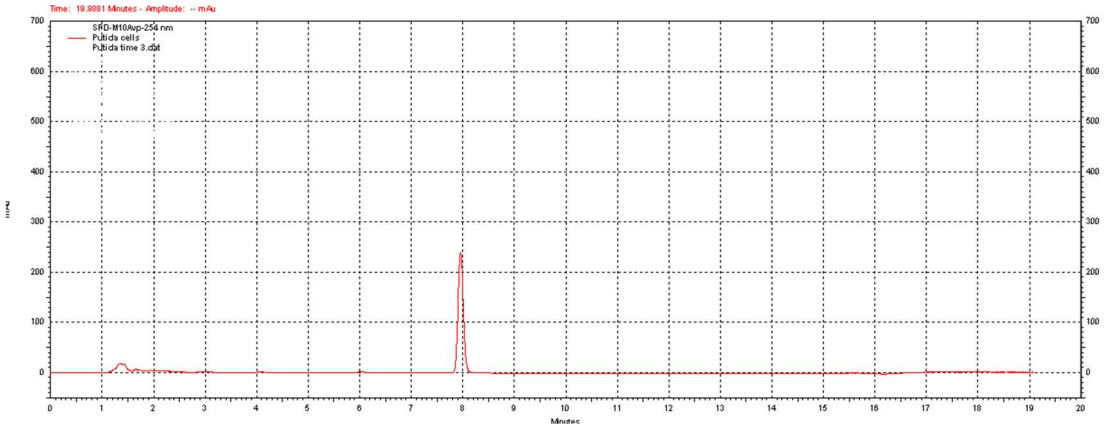

Figure 109: Chromatogram after 3 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

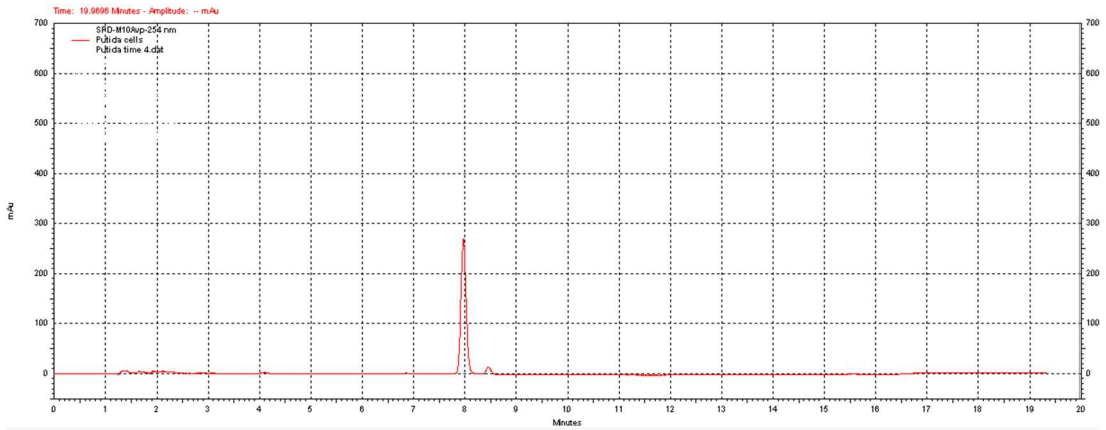

Figure 110: Chromatogram after 4 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

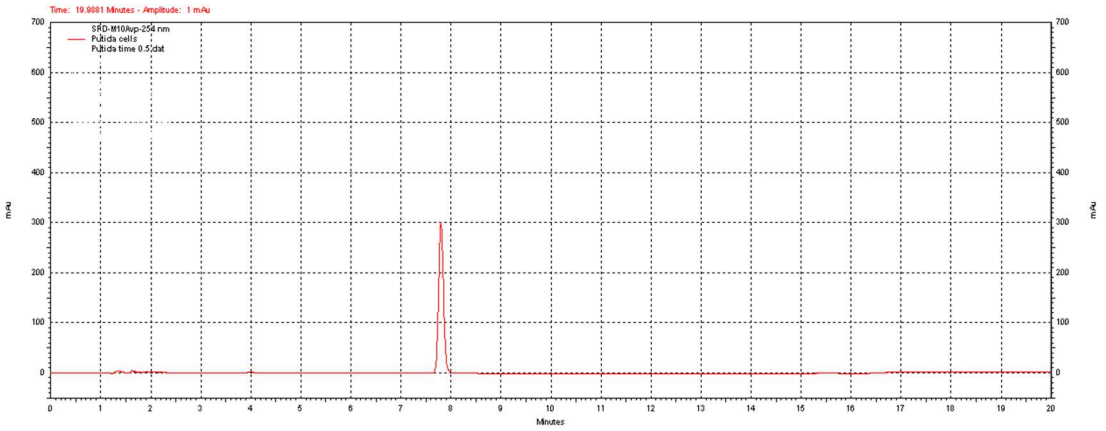

Figure 111: Chromatogram after 0 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

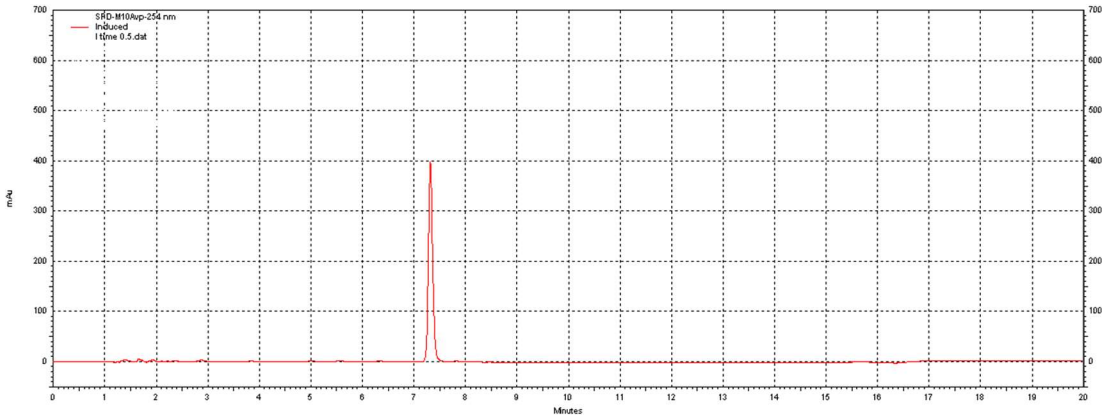

Figure 112: Chromatogram after 0.5 hour of incubation of CBDA with lysed *P. putida.* Induced 3 days with 10 mM salicylic acid.

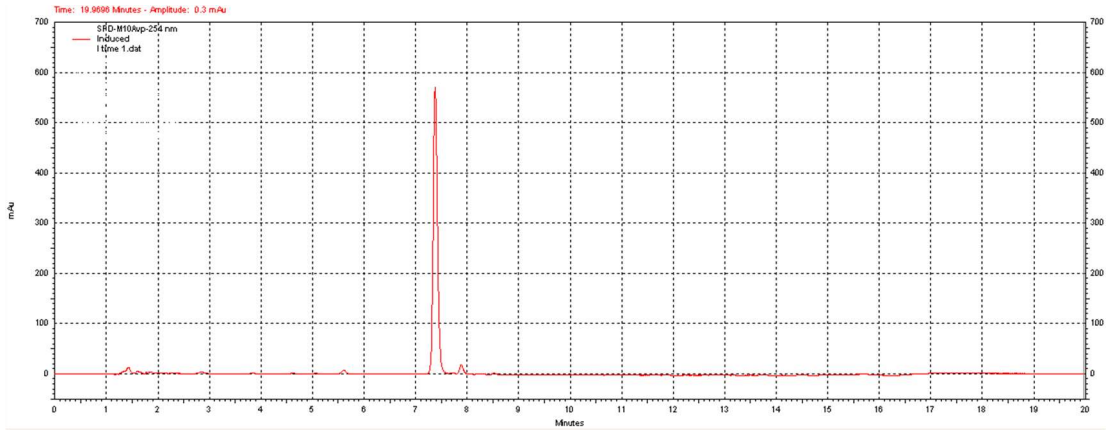

Figure 113: Chromatogram after 1 hour of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

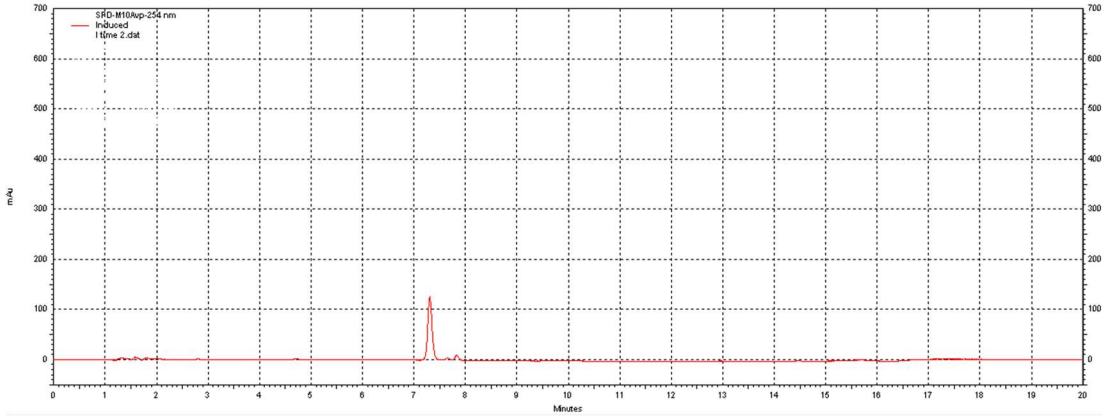

Figure 114: Chromatogram after 2 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

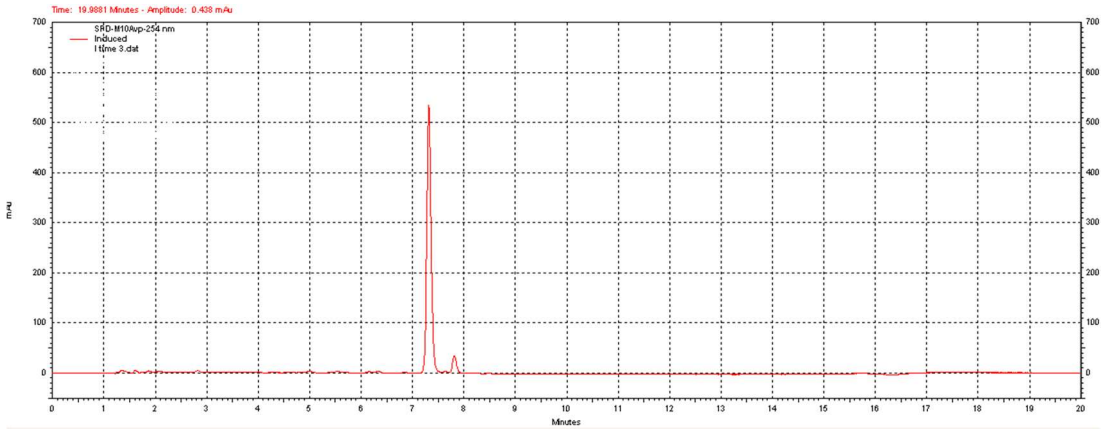

Figure 115: Chromatogram after 3 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

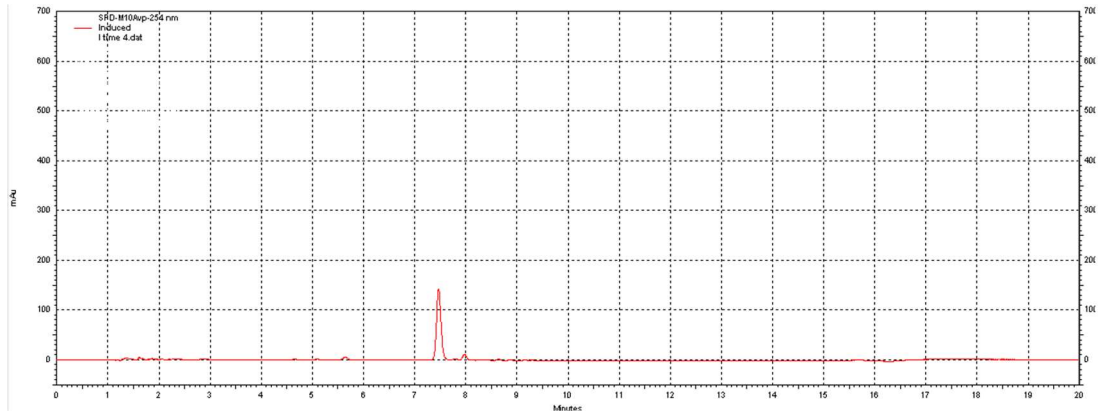

Figure 116: Chromatogram after 4 hours of incubation of CBDA with lysed *P. putida* induced with 10 mM salicylic acid.

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